University of Alberta

Control of Floral Induction in *Brassica napus*

By

Nancy Erro Go ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science in Plant Science

Department of Agricultural, Food and Nutritional Science

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Nancy Go

Nancy Go
17062-100 St.,
Edmonton, AB T5X 5E1

Date: Nov 23, 2000

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Control of Floral Induction In *Brassica napus* submitted by Nancy Erro Go in partial fulfillment of requirements for the degree of Master of Science in Plant Science.

Dr. Anne M. Johnson-Flanagan

Dr. Gary Stringam

Dr. Dave Cass

Date: NOV 13, 2000

ABSTRACT

The shift from vegetative to reproductive growth is influenced by environmental conditions with light and temperature as the major controlling factors. In this study, the flowering responses of several *B. napus* spring lines to photoperiod and vernalization were characterized in order to determine the mechanisms controlling floral induction in this species. The *B. napus* spring lines were able to flower under 16-hr and 8-hr photoperiods and their flowering responses were accelerated by vernalization. This suggests that the floral induction pathways identified in *Arabidopsis* also exist in the *B. napus* spring lines and hence, the genetic mechanisms controlling floral induction in early-flowering ecotypes of these two species may be similar. It was also shown that in *B. napus* spring lines, the light dependent pathway is the major pathway regulating floral induction and the vernalization response is dependent on the photoperiod conditions. Thus, the vernalization response of spring lines must always be analyzed and interpreted with reference to photoperiod conditions.

Results obtained from this study indicate that the genetic mechanisms involved in the cold induction of flowering are different between *B. napus* winter lines that have an obligate vernalization requirement and *Arabidopsis* late flowering ecotypes that have a facultative vernalization requirement. The *B. napus* winter lines 6-202 and Cascade were not able to flower without vernalization and once vernalized, showed no photoperiodic response. The obligate vernalization requirement in these *B. napus* winter lines was due the presence of the Flowering Locus C (FLC) gene product, a non-functional light regulated pathway and possibly, defects in the constitutive pathway as well. Previous studies on *Arabidopsis* late-flowering ecotypes have shown that FLC is involved both in conferring the vernalization requirement and in the quantitative flowering

response to vernalization. In the *B. napus* winter line 6-202, FLC is involved only in conferring the vernalization requirement. Overcoming FLC repression of flowering by vernalization in this plant system is an all or nothing process. It is suggested that other gene(s) downstream of FLC may be responsible for the quantitative flowering response to vernalization in Line 6-202.

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ABBREVIATIONS

ANOVA Analysis of Variance
DAP Days after Planting
DAV Days after Vernalization
DH Double Haploid
DNA Deoxyribonucleic Acid
EDTA Disodium Ethylenediaminetetraacetic Acid
FLC Flowering Locus C
GAs Gibberellins
GLM General Linear Model
gm/gms gram/grams
hr/hrs hour/hours
LD Long Day
LG Linkage Group
M Molar
MADS Box region of homologous sequences found in transcription factors involved in flowering (Minichromosome Maintenance Gene, AGAMOUS, DEFICIENS, Serum)
min/mins minute/minutes
mM millimolar
ml/mls milliliter/milliliters
mRNA messenger Ribonucleic Acid
ng/ml nanogram per milliliter
QTL Quantitative Trait Loci
RFLP Restriction Fragment Length Polymorphism
RNA Ribonucleic Acid
RNAse Ribonuclease
rpm revolutions per minute
SAS Statistical Analysis Software
SD Short Day
SDS Sodium Dodecyl Sulfate
SEM Scanning Electron Microscopy

U Units

µL microliter

µM micromolar

µg microgram

V Volts

VFN1/VFN2 Vernalization-responsive Flowering Time Loci in *B. napus*

wk/wks week/weeks

CHAPTER I

INTRODUCTION

Plant species insure the success of their reproductive growth by flowering at the same time under favorable growing conditions. Plants are able to do this through the recognition of the appropriate environmental signals, mainly photoperiod and temperature. The process is known as floral induction/evocation and it starts when the external or environmental cues are perceived and converted into internal biochemical signals. These internal signals then redirect the mode of meristem growth and differentiation from the vegetative to the reproductive states. The process ends with the formation of an inflorescence meristem that will eventually give rise to the flowers. The first hypothesis that tried to explain the physiological basis of floral induction proposed that the process is mediated by the balance of two hormones termed "florigen", which promotes flowering, and "anti-florigen", which inhibits flowering. Sixty-three years have passed since this hypothesis was proposed and research done over the years has shown that induction of flowering may not be as simple as a result of the antagonistic actions of two substances. It appears that it is a complex developmental program governed by a number of internal factors whose effects vary with different plant genotypes. Although significant progress has been made in identifying these factors, a complete picture on the physiological and genetic mechanisms controlling floral induction remains unclear.

Plants can be classified into two groups according to their environmental cues for flowering. Photoperiodic plants flower based on daylength requirement. The other group consists of plants that flower only when exposed to cold temperatures, a process known as vernalization. In a number of plant species, both photoperiod and cold temperature can induce flowering. Other plants depend not on environmental cues but on developmental age. Based on these flowering requirements, it has been proposed that there are three pathways involved in floral induction. Recent genetic analyses in *Arabidopsis* indicate that this might be the case. However, how these pathways function is still not clearly understood.

This study was focused on floral induction in *Brassica napus*, an important crop in Canada. In *B. napus*, two flowering genotypes exist. Spring or annual types are quantitative long day plants whereas winter or biennial types do not flower without a cold treatment. Due to the restrictions imposed by the vernalization requirement in winter lines, most breeding programs have been limited to inter-crossing within groups. Hence, there is an increasing interest to utilize the genetic resources found in winter lines to increase the genetic diversity in spring cultivars and vice-versa. The difference in flowering requirement, however, imposes a major limitation and slows down the introgression process. Therefore, there is a need to understand the genetic mechanisms by which flowering is initiated in spring and winter lines.

This study is divided into two parts. The first part deals mainly with the characterization of the flowering responses of several spring and winter lines of *B. napus* and genetic analysis of these lines using RFLP markers linked to flowering time and vernalization requirement. The second section focuses on the characterization of the vernalization responses in the winter line, 6-202 and expression of FLC (Fowering Locus C) in relation to these responses. FLC has been proposed to be the central gene involved in the vernalization response in *Arabidopsis*.

CHAPTER II

REVIEW OF LITERATURE

1. Overview

Reproductive growth is divided into two phases. The floral induction phase comprises all processes leading to the transformation of the vegetative meristem into an inflorescence meristem. The second phase, floral morphogenesis, includes all subsequent processes leading to the formation of the floral meristem and eventually, the floral organs. These two phases are two separate events involving two different sets of control factors (Huala & Sussex 1993, Haughn et al. 1995).

The mechanisms involved in floral induction had been the subject of numerous studies for a number of decades (Bernier 1988). Early studies centered mostly on elucidating the physiological basis of floral induction. In recent years, however, studies have focused on the genetic mechanism underlying this process (Koornneef et al. 1998, Levy & Dean 1998, Pineiro & Coupland 1998, Simpson et al. 1999). Much of the progress in this area has been facilitated by isolation of flowering mutants of *Arabidopsis*. Based on the number of genes involved in regulating flowering time as well as the different responses of the mutants to light and temperature, floral induction in *Arabidopsis* is thought to be under multifactorial control. Research done on other plant species such as pea also indicates multigenic control of flowering (Poethig 1990).

Characterization of the flowering responses of the *Arabidopsis* flowering-time mutants to photoperiod and vernalization have led to the identification of at least three pathways controlling floral induction in this system (Koornneef et al. 1998, Levy & Dean 1998). The photoperiodic induction pathway involves long day promotion of flowering whereas the constitutive or autonomous pathway allows flowering to proceed under short day conditions and is probably determined by the physiological or developmental state of the plant. Lastly, the vernalization pathway involves promotion of flowering by exposure to cold temperatures.

2. Meristem Competence

Plants grown under inductive flowering conditions do not proceed to reproductive growth as soon as they germinate but instead, go through a minimum period of vegetative growth. The vegetative period is divided into two phases, i.e., the juvenile phase in which the meristem is not capable of responding to floral stimuli and the adult phase wherein the meristem acquires the competence to do so (Poethig 1990, McDaniel et al. 1992). The shift from juvenile to adult vegetative phase is usually associated with morphological changes in the plant, the most conspicuous of which are changes in leaf shape, size and phyllotaxy. In *Arabidopsis*, for example, the rosette leaves formed early in the vegetative phase are small, round and entire whereas those formed later are large, spatulate and serrated (Haughn et al. 1995). These changes in leaf morphology are also accompanied by a shift from opposite to spiral phyllotaxy. In *Zea mays*, anatomical alterations are also observed such as changes in epidermal cell shape and cuticle thickness and absence or presence of epicuticular wax, epidermal hairs and bulliform cells (Poethig 1990).

Very little is known about the physiological mechanisms controlling the juvenile to adult vegetative phase transition. Most experiments designed to determine the biochemical factors controlling this phase change involved grafting scions with meristems at the juvenile state onto mature flowering stocks and assessing flowering response (Bernier 1988, Poethig 1990, McDaniel et al. 1992). The results varied with different plant species. In most woody species, juvenile scions did not flower more rapidly when grafted onto mature flowering stocks. This indicates that there are inherent factors in the meristem that determine its competence to respond to a floral stimulus or stimuli. However, there are certain exceptions to this, e.g., in fig and pecan, flowering was hastened under the said conditions (Bernier 1988). Herbaceous species, on the other hand, showed the opposite response. Juvenile scions from most herbaceous plants readily flowered when grafted onto flowering stocks (Bernier 1988). This implies that the juvenile or adult state of the meristem is determined by physiological factors coming from other plant parts. Again, not all results support this hypothesis. For example, scions from different varieties of *Xanthium strumarium* flowered differently when grafted onto the same stock (Carpenter & Lincoln 1959). Likewise, in *Nicotiana*,

scions with ontogenically older meristems flowered earlier as compared to scions with younger meristems (McDaniel et al. 1992). On the other hand, in *Bidens radiata*, the ability to respond to photoperiodic induction decreases with plant age (Pouhle et al. 1984). Poethig (1990) suggested that the vegetative phase transition is initiated by factors extrinsic to the meristem but there are intrinsic mechanisms in the meristem that determines its ability to respond to these extrinsic factors. Apparently, the extent at which these factors exert their effect varies with different plant genotypes.

Only a few genes have been specifically identified to act in the juvenile to adult vegetative phase transition. In maize, Poethig (1988) found three semi-dominant genes regulating shoot growth: TP1 (TEOPOD 1), TP2 and TP3. The TP mutants have been characterized as having gain-of-function mutations, which indicates that the TP genes are promoters of juvenile vegetative growth. In *Arabidopsis*, the HASTY gene has been identified as a promoter of juvenile growth (Telfer & Poethig 1998). A repressor of juvenile growth has been isolated in *Arabidopsis*, FPF1 (FLOWER PROMOTING FACTOR 1) and it encodes a novel protein that may be involved in signaling or response to gibberellins (Kania et al. 1997, Melzer et al. 1999). Another mutant, *eaf1* (*early flowering 1*), which shows shortened juvenile phase, has also been implicated in the GA regulation of flowering (Scott et al. 1999).

3. Photoperiodic Induction of Flowering

3.1. Perception of Light Signal

Perception of inductive light conditions takes place primarily in mature leaves (Havelange & Bernier 1991, McDaniel et al. 1991) although there have been reports that very young leaves and shoot apices are also sensitive to photoperiodic treatments (Kinet 1993). Plants that flower or have accelerated flowering in long days or short days are known as LD and SD plants, respectively. Although the name implies light requirement, it is actually the night length that controls the flowering response to photoperiod as shown by the "night break" phenomenon. Light flashes during the dark period will cause SD plants to remain vegetative whereas LD plants will be induced to flower. The presence of SD and LD plants and fluctuations in the light sensitivity of these plants indicate that aside from photoreceptors detecting the presence or absence of light, an

endogenous circadian clock is also involved in the photoperiodic control of flowering (O'Neill 1992, Perilleux et al. 1994). It has been hypothesized that these two systems control floral induction by regulating each other (Lin 2000). Photoreceptors directly mediate the signaling mechanisms that regulate floral initiation and they also synchronize the circadian clock with environmental light conditions. The circadian clock, in turn, controls the photoreceptor-mediated signal transduction by allowing or denying it to proceed at certain times in the cycle.

3.1.1. Photoreceptors

There are two primary photoreceptors in higher plants (Lin 2000, Ma 1998). The first group consists of phytochromes that are photochronic proteins absorbing light in the red/far-red end of the spectrum (600-700 nm). They exist in two reversible isomeric forms: Pr absorbs red-light and is converted into Pfr whereas Pfr absorbs far-red light and is converted back to Pr. Exposure to red light leads to delayed flowering and thus, the activated Pfr form is inhibitory to floral induction (Goto et al. 1991, Bagnall 1992, 1993). The second group of photoreceptors is made up of flavoproteins called cryptochromes that are able to detect blue light (400-500 nm). Blue light accelerates the shift to reproductive growth (Eskins 1992).

In *Arabidopsis*, five phytochrome genes (PHYA to E) have been isolated and sequenced (Sharrock & Quail 1989, Clack et al. 1994). Sequence analysis showed that PHYB, PHYD, and PHYE have 80% amino acid similarity and they all function as inhibitors of floral induction (Devlin et al. 1998, 1999; Neff & Chory 1998). In contrast, PHYA acts as a promoter of floral initiation (Neff & Chory 1998) and shows a 65-80& homology to Type I phytochrome apoproteins. Previous physiological work on phytochromes showed that there are two types (O'Neill 1992). Type I phytochromes are relatively abundant in etiolated tissues and light-labile in the Pfr form. The other type of phytochrome, Type II, is present in low abundance in green tissue and light-stable in the Pfr form. Based on the inhibitory nature of PHYB, PHYD and PHYE, they may comprise the Type II phytochromes. Two genes encoding *Arabidopsis* cryptochromes have been isolated; CRY1, previously known as HY4 (Ahmad & Cashmore 1993, Bagnall et al. 1996) and CRY2 (Guo et al. 1998). Analysis of multiple phytochrome and cryptochrome mutants revealed that interactions exist between these photoreceptors (Lin 2000).

PHYB, PHYD and PHYE have redundant functions in directly mediating red-light inhibition of floral induction. PHYA and CRY2 promote floral induction by suppressing the effect of these inhibitory phytochromes. CRY2, together with CRY1, can also directly mediate blue-light promotion of flowering.

How photoreceptors initiate the signal transduction pathway for floral induction is still unclear. There have been reports that phytochromes can act as protein kinases (Frankhauser et al. 1999) or they can interact with other nuclear proteins that modulate expression of light-regulated genes (Ni et al. 1998). Ahmad and Cashmore (1996) isolated early-flowering *Arabidopsis* mutants, *pef1* (*phytochrome-signaling early-flowering*), *pef2*, *pef3*, and these loci might code for proteins involved in the early steps of phytochrome signaling pathway. Lin (2000) suggested that since the photoreceptors are soluble proteins and can enter the nucleus, they could also directly regulate expression of flowering-time genes. Indeed, levels of CO (CONSTANS) have been shown to increase in transgenic plants over-expressing CRY2 and decrease in *cry2* mutants lacking the functional cryptochrome (Guo et al. 1998).

3.1.2. Circadian Clock

A basic circadian clock system has three primary components (Somers 1999). First is the central oscillator/pacemaker that generates the 24-hr oscillations. In animals and and cyanobacteria, this is made up proteins whose interactions create an autoregulatory negative feedback loop which, in turn, generates the 24-hr oscillations. In plants, it is still unknown how the oscillator operates but four genes that affect flowering response had been identified as potential oscillator elements. Mutations in these genes delay flowering and/or abolish flowering time differences under LD and SD conditions. Two of these genes, CCA 1 (CIRCADIAN CLOCK ASSOCIATED 1) and LHY (LATE ELONGATED HYPOCOTYL), have been sequenced and shown to encode MYB DNA-binding proteins (Wang et al. 1997, Wang & Tobin 1998, Schaffer et al. 1998). GI (GIGANTEA) codes for a putative membrane protein (Lin 2000). The identity of other gene, TOC1-1, remains unknown (Somers et al. 1998).

The second component is the input pathway that synchronizes or entrains the oscillator with environmental cues. In light-coordinated circadian clock, this function is

carried out by the photoreceptors (Millar et al. 1995, Somers et al. 1998). Phytochrome B is the primary photoreceptor for high intensity red light whereas phytochrome A acts under low intensity red light. Cryptochrome 1 and phytochrome A both act to relay low-fluence blue light to the clock. Analysis of the *Arabidopsis* early-flowering mutants *cop1* (*constitutive photomorphogenic 1*), *det1* (*de-etiolated 1*) and *elf3* (*early-flowering 3*) showed that the disrupted genes might also be part of the input pathway (Deng et al. 1991, Hicks et al. 1996, Kwok et al. 1996). COP1 encodes a protein that has three structural domains with associated with transcription factors; a zinc-binding motif, a potential coiled-coil region and a Gβ-protein homologous sequence (McNellis et al. 1994). DET1 (DE-ETIOLATED 1) encodes a novel nuclear-localized protein and it may control cell-type specific expression of light regulated promoters (Pepper et al. 1994).

The third is the output pathway that links the oscillator to plant processes under circadian rhythm. Unfortunately, not very much is known about the molecular mechanisms connecting the circadian clock to floral induction.

3.2. Biochemical Signals

The spatial separation between where the light signal is received (leaves) and where the reproductive structures will eventually form (shoot apex) indicates the presence of transmissible flowering signals. Three hypotheses have been forwarded to explain the chemical nature of these signals. Based on grafting experiments, the "florigen/antiflorigen" theory involves the presence of two universal promoter and inhibitor hormones, though no such hormones have been isolated or identified so far (Zeevaart 1984, Schwabe 1984). The "nutrient diversion" concept proposes that under inductive conditions, assimilates are preferentially transported to the shoot apex and this increase in nutrient level serves as a trigger for reproductive growth (Bodson 1984). Lastly, the "multifactorial control" hypothesis suggests that floral induction involves different chemical factors and that they should be present and the apex at appropriate levels and at the right developmental and physiological conditions to evoke flowering (Bernier 1988, Bernier et al. 1993). Further, the limiting factor depends on the plant genotype and/or environmental conditions. Based on studies with *Arabidopsis* which show a multigenic control of floral induction, the "multifactorial control" seems to be the most plausible hypothesis (Koornneef et al. 1991).

Physiological studies either through exogenous application or analysis of extracts from induced and non-induced plants have identified several factors that either inhibit or promote flowering (Bernier 1988, Bernier et al. 1993, Kinet 1993). Most of these factors are carbohydrates and plant growth regulators such as cytokinins, auxins, abscisic acid, ethylene and gibberellins. The effect of the plant growth regulators varies depending on the plant species but in general, cytokinins have promotive effects while auxins, ABA and ethylene inhibit flowering. *Arabidopsis* mutants defective in the synthesis or response to these hormones support these physiological findings. ABA mutants, *aba1* (*ABA deficient 1*) and *abai* (*ABA insensitive*), are early flowering (Lee et al. 1998). The role of ethylene is not clear since mutants not responsive to ethylene showed late-flowering phenotypes (Martinez-Zapater et al. 1994). A mutant with high levels of cytokinins, *amp1* (*altered meristem 1*), is both early flowering and day-length insensitive which suggest that cytokinins may be involved in the light signal transduction (Chaudhury et al. 1993). Another group of hormones, the brassinosteroids, has been implicated in floral induction. DET2 (DE-ETIOLATED 2) encodes steroid 5a-reductase, an enzyme involved in brassinolide biosynthesis. Lesion in this gene results to late flowering (Chory et al. 1991, Fujioka et al. 1997).

Among the plant hormones, the GAs are the most effective in eliciting flowering response in LD plants grown under non-inductive conditions. However, the flowering response varies depending on the plant species and forms of GAs used (Pharis & King 1985, Pharis 1991). It has been observed that plants belonging to certain groups have uniform responses to GA application. For example, in conifers, species belonging to Taxodiaceae and Cupressaceae will initiate conebuds when treated with any bioactive GAs whereas conifers in the Pinaceae will only respond to less polar GAs. In LD plants, it appears that highly florigenic GAs have a structure consisting of 19 carbons with: (1) a carboxylated C-7; (2) a ring A that has a double bond between C-1 and C2 or C-2 and C-3 and; (3) a ring C that is hydroxylated (C-13) or ideally, polyhydroxylated rings C and D (C-12, C-13 and/or C-15) (Pharis et al. 1992). The highly florigenic GAs are normally inhibitory to stem elongation whereas GAs promoting stem elongation have low florigenic activities. In short-day plants, the presence of florigenic GAs has not been established. Two *Arabidopsis* GA mutants, *ga1* and *gai* (*GA insensitive*), exhibit extreme late-flowering phenotypes only in SD but not in LD (Wilson et al. 1992). This

suggests that GA induction of flowering may not be through the light-controlled pathway. Indeed, Putterill et al. (1995) found that supplying GA to *co*, a mutant that is defective in a gene involved in the light-dependent pathway, does not correct the late-flowering phenotype exhibited by this mutant. The GA1 locus codes for cyclase ent-kaurene synthetase A or copalyl diphosphate synthase which catalyzes the conversion of geranylgeranyl pyrophosphate to copalyl pyrophosphate, the first committed step in the GA biosynthetic pathway (Sun & Kamiya 1994). Deficiency in this enzyme has been correlated with decreased expression of LEAFY, a gene involved in establishing floral meristem identity (Blazquez et al. 1998). The early-flowering mutant *spy* (*spindly*) has a defective gene that codes for the enzyme O-linked N-acetylglucosamine transferase, which modifies proteins (Levy & Dean 1998). SPY is probably involved in the GA signal transduction pathway (Jacobsen & Olszewski 1993).

There is increasing evidence that sucrose may have a regulatory function in floral induction, other than providing energy for the flowering process. Sucrose concentrations in the leaves and leaf exudates as well as in the apical meristems have been shown to increase rapidly in LD and SD plants after exposure to inductive light conditions (Bodson & Outlaw 1985, Lejeune et al. 1991, Houssa et al. 1991, Lejeune et al. 1993). Roldan et al. (1997) grew late-flowering ecotypes of *Arabidopsis* in culture media allowing direct availabilty of sucrose to the apical meristems. This resulted in significant reduction in final leaf number. Moreover, tomato plants transformed with sucrose phosphate synthase and hence, with increased capacity to synthesize sucrose, had shorter flowering times compared to wild-type controls (Micallef et al. 1995). Finally, several mutants defective in starch/sucrose metabolism (*pgm*, *adg1*, *sex1*, *cam1*) exhibited late-flowering phenotypes which can be rescued by vernalization, indicating that the late-flowering response is not a result of slow growth but by a delay in floral transition (Bernier et al. 1993). A mutant, *sun2* (*sucrose-uncoupled 2*), characterized by reduced sucrose repression of plastocyanin and chlorophyll a/b binding proteins synthesis also showed an early-flowering phenotype (Dijkwel et al. 1997). This suggests an interaction between sucrose and light signaling in the flowering process.

3.3. Flowering Time Specific Genes

Most of the genes that have been described above have pleiotropic effects affecting other light-dependent developmental and physiological processes aside from flowering (Hicks et al. 1996, Levy & Dean 1998, Somers 1999). The action of these genes, therefore, constitutes the early and probably common steps in the pathways regulating light-mediated plant processes. These pathways diverge at certain points becoming more specific and as such, several genes that act solely to affect flowering time have been identified.

Flowering time mutants of *Arabidopsis* are either early- or late-flowering indicating the presence of genes that repress or promote floral induction, respectively (Amasino 1996, Levy & Dean 1998, Koornneef et al. 1998, Pineiro & Coupland 1998). So far, no non-flowering mutants have been isolated and this suggests that genes promoting flowering have redundant functions, i.e., other genes can compensate for the inactivation of one gene (Pineiro & Coupland 1998).

The loci involved in the promotion of flowering are CO, FD, FE, FT and FWA. Mutants in these genes show no photoperiodic and limited vernalization responses (Bagnall 1993, Martinez-Zapater & Somerville 1990). Two of these genes have been cloned and sequenced. CO (CONSTANS) encodes a protein showing similarities to zinc finger transcription factors (Putterill et al. 1995) while the deduced amino acid sequence of FT (FLOWERING LOCUS T) has the highest homology to phosphatidylethanolamine-binding proteins (Levy & Dean 1998). Ma (1998) proposed that CO, which is highly expressed in leaves in LD but not in SD, may be the gene controlling the production of transmissible flowering signals from the leaf to the apex. Constitutive expression of CO results in early flowering and abolishes flowering time differences under LD and SD conditions (Coupland 1998). A similar zinc-finger protein, ID (INDETERMINATE), has been identified in maize and it also regulates a leaf-generated flowering signal (Colasanti et al. 1998). Recent genetic analysis (Onouchi et al. 2000), however, indicates that CO requires FT and FWA to promote floral induction.

To date, thirteen genes (TFL1, EMF1, EMF2, CLF, WLC, ELF1, ELF2, ELG, PIF, EFS, EBS, ESD4, WLC) have been identified that repress floral induction (Levy & Dean

1998, Koornneef et al. 1998). Unlike the genes involved in the promotion of floral induction, mutations in these genes have pleiotropic effects influencing leaf, flower and seed morphology and overall plant size (Hicks et al. 1996). It is, however, not clear if these effects are directly caused by these genes or a consequence of the shortened vegetative phase. The CLF gene (CURLY LEAF) shows homology to *Drosophila* polycomb genes, which are involved in the repression of homeotic genes (Levy & Dean 1998). Both WLC (WAVY LEAVES AND COTYLEDONS) and ESD4 (EARLY IN SHORT DAYS 4) encode novel proteins (Levy & Dean 1998). The TFL1 (TERMINAL FLOWER 1) putatively encodes a phophotidylethanolamine-binding protein (Oshima et al. 1997). The mutant phenotypes of TFL1, EMF1 and EMF2 provide insight into the important function that these genes play in regulating the vegetative-reproductive phase transition. Instead of an inflorescence, the *tfl1* mutant produces a few flowers that have anomalous organ formation. It is also photoperiod insensitive (Hicks et al. 1996). Thus, TFL1 provides a link between flowering-time and floral meristem identity genes. Mutations in the EMF (EMBRYONIC FLOWERING) genes have similar but more severe morphological abnormalities such as sessile rosette leaves and flowers that lack petals and anthers (Sung et al. 1992). These defects can be traced back to the embryonic stage wherein the shoot apical meristem does not produce the typical tunica corpus (Bai & Sung 1995). It has been proposed that blocking EMF function results in the development program progressing directly to the reproductive phase, completely bypassing the vegetative phase (Yang et al. 1995). In wild-type plants, EMF expression is presumed to be high during the early stages of growth, decreasing as the plants age and may be eliminated by long-day conditions.

4. Vernalization

4.1. Perception of Cold Temperature

Perception of cold temperatures occurs in the shoot tip which includes the apex and immature leaves (Metzger 1988). However, there are reports that immature leaf and root explants cultured in vitro can regenerate flower-forming shoots. This shows that dividing cells from any plant organ can respond to vernalization (Metzger 1988, Burn et al 1993). It is still not understood how the cold signal transduction pathway is mediated since no thermoreceptor has been identified. Manabu et al. (1998) identified a gene,

HOS1, that acts as negative regulator of cold signal transduction. Lesions in this gene result in enhanced expression of the cold-responsive genes and early-flowering and thus, may function in the early steps of the cold signaling pathway.

4.2. Biochemical Signals

Gibberellins induce flowering in a number of vernalization-requiring plants and hence, had been the focus of most studies dealing with biochemical signals in vernalization (Bernier 1988, Kinet 1993). Working with *Thlaspi arvense*, Hazerbroek et al. (1993) noted a cold-induced increase in the turnover of kaurenoic acid to 7β-hydroxy kaurenoic acid, a reaction which is catalyzed by kaurenoic acid hydroxylase. This increase was restricted to the shoot tips where cold temperature perception occurs. Burn et al. (1993) also found that non-vernalized *T. arvense* plants could be induced to flower when treated with DNA demethylating agents. Based on these results, it has been hypothesized that cold temperatures reduce the methylation status of genes. Vernalization occurs when the gene encoding kaurenoic acid hydroxylase is demethylated thereby allowing its transcription to proceed (Dennis et al. 1996). Finnegan et al. (1998) reported that *Arabidopsis* C24 plants transformed with an anti-sense methyltransferase gene flowered earlier than wild-type plants without vernalization, thus, further corroborating this hypothesis. However, Ronemus et al. (1996) have shown that in a different *Arabidopsis* genetic background (Col), demethylation resulted in late flowering. The role of GA in vernalization is still speculative since Michaels & Amasino (1999) found that *Arabidopsis ga1* mutations do not block the response to vernalization under LD conditions. They suggested that GA may not have a direct role in vernalization but is required in an alternate pathway that promotes flowering in noninductive photoperiods.

4.3. Flowering Time Specific Genes

To date, only a few genes involved in the vernalization pathway in *Arabidopsis* have been reported. This is to be expected since all the ecotypes that have been studied are early- or late-flowering wherein floral induction is mainly controlled by photoperiod and vernalization merely hastens the flowering response. As such, it is difficult to screen for mutants affected specifically in the cold-regulated pathway. Two genes involved in the

vernalization response have been isolated in the *fca* mutant background (Chandler et al. 1996). The VRN1 and VRN2 loci were isolated from the late-flowering, vernalization responsive *fca* mutant on the basis that mutations in these genes resulted in late-flowering even after vernalization treatment. These genes, therefore, are involved in the vernalization response. As such, they might act in the perception of cold temperature or transduction of the signal (Koornneef et al. 1998). However, they are specific for vernalization since the mutations did not affect expression of other cold-regulated genes (Chandler et al. 1996).

Genetic analyses of naturally occurring early- and late-flowering ecotypes of *Arabidopsis* have identified several genes influencing flowering-time. Genes with minor effects are JUV (JUVENALIS), KRY (KRYOPHILA) and ART (AERIAL ROSETTE) (Koornneef et al. 1998). The two genes that account mostly for the variation in flowering time in Arabidopsis are FRI (FRIGIDA) and FLC (FLOWERING LOCUS C) (Lee et al. 1993, Clarke & Dean 1994, Lee et al. 1994, Aukerman & Amasino 1996, Sanda et al. 1997). These two act synergistically to cause late flowering and dominant alleles in both loci cause extreme lateness. The late-flowering phenotype conferred by these five genes can be reversed by vernalization. The gene product of FLC has been identified and found to be involved in the vernalization response (Michaels & Amasino 1999, Sheldon et al. 1999).

The FLC (=FLF) gene, which encodes a novel MADS-domain transcription factor, was identified both through allelic variation (Michaels & Amasino 1999) and mutant analysis (Sheldon et al. 1999). FLC confers vernalization requirement and at the same time, is also involved in the response to vernalization. Increased expression of FLC leads to delay in flowering in late-flowering ecotypes as well as in late-flowering mutants (Sheldon et al. 1999). The down regulation of FLC by cold temperature is proportional to the duration of the treatment and to the resulting decrease in flowering time (Sheldon et al. 2000). With these observations, FLC is thought to be the central gene involved in the regulation of flowering by vernalization.

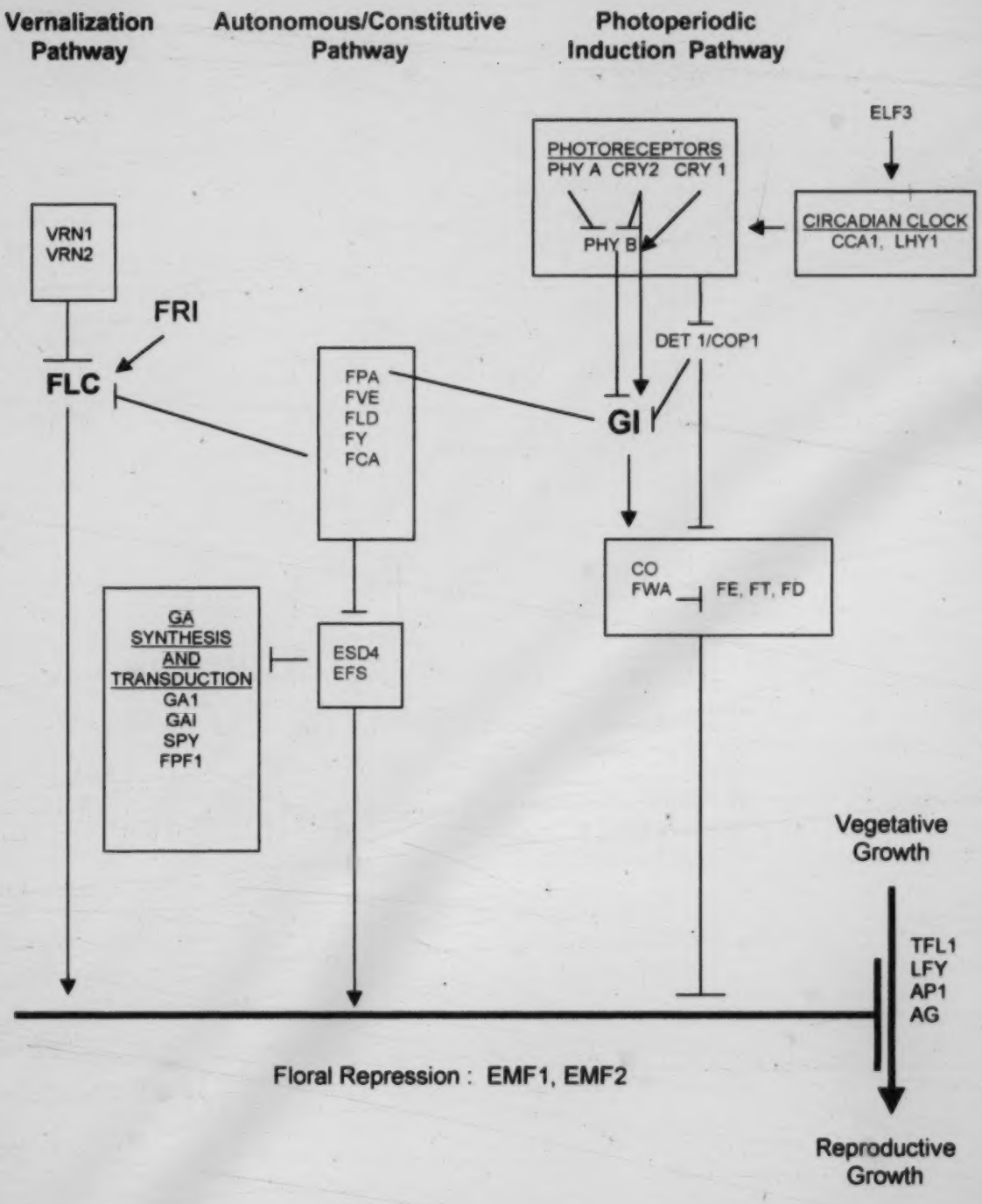
5. Autonomous Induction of Flowering

A group of late-flowering mutants is characterized by strong photoperiodic and vernalization responses (Martinez-Zapater et al. 1994). The affected genes, FCA, FPA, LD, FVE, FY and FLD, constitute the autonomous or constitutive (light-independent) promotion pathway. As the mutants also have short internodes, it has been proposed that these genes are involved in the constitutive regulation of GA biosynthesis and/or GA sensitivity (Martinez-Zapater et al. 1994). Only two of these genes have been identified and they function as transcriptional regulators. The predicted amino acid sequence of LD (LUMINIDEPENDENS) has two bipartite nuclear localization signals and a glutamine-rich domain that is characteristic of certain transcription factors (Lee et al. 1994). FCA, on the other hand, encodes a protein with RNA binding and protein-protein binding domains which suggests that it is involved in post-transcriptional regulation of transcripts (Macknight et al. 1997). Repressor genes in this pathway have also been identified. An early-flowering mutant, *tfl2*, showing no photoperiodic response but similar morphological abnormalities in *tfl1*, provides a link between autonomous floral induction and floral morphogenesis (Hicks et al. 1996). Another early-flowering mutant is *efs* (*early flowering in short days*) which is not responsive to vernalization (Soppe et al. 1999). It has been suggested that EFS may also be a component of the vernalization pathway.

6. Working Model of the Genetic Mechanisms Regulating Floral Induction

In *Arabidopsis*, a significant number of genes involved in floral induction have already been identified. Based on the responses of the mutants to photoperiod and vernalization, it has been established that there are at least three pathways by which floral induction is regulated. Further phenotypic characterization of double or triple mutants has identified genetic interactions among these flowering time genes. These results have led to the construction of a model showing the genetic mechanisms by which these genes regulate the shift from vegetative to reproductive growth. This is summarized in Figure 1 (Koorneef et al. 1998, Simpson et al. 1999, Lin 2000). At the center of the three pathways are the EMF genes that act to repress the shift to reproductive growth. The flowering-time specific genes regulate floral induction by

Figure 1. A model showing the genetic mechanisms involved in the regulation of floral induction in *Arabidopsis*. Genes involved in the three pathways of floral induction are indicated by their colors: blue (vernalization pathway), red (autonomous pathway) and green (photoperiodic induction pathway). Promotive interactions between the genes or groups of genes are indicated by "⟶" and repressive interactions by "⊣". (Adapted from Koornneef et al. 1998, Simpson et al. 1999, Lin 2000)


Vernalization Pathway
Autonomous/Constitutive Pathway
Photoperiodic Induction Pathway
ELF3
PHOTORECEPTORS
PHY A CRY2 CRY 1
PHY B
CIRCADIAN CLOCK
CCA1, LHY1
VRN1
VRN2
FRI
FLC
DET 1/COP1
FPA
FVE
FLD
FY
FCA
GI
CO
FWA
FE, FT, FD
GA SYNTHESIS AND TRANSDUCTION
GA1
GAI
SPY
FPF1
ESD4
EFS
Vegetative Growth
TFL1
LFY
AP1
AG
Floral Repression : EMF1, EMF2
Reproductive Growth

inhibiting or promoting expression of EMF. These genes, in turn, are regulated by genes involved in signal perception and transduction.

The link between factors controlling floral induction and floral morphogenesis are the genes involved in maintaining floral meristem identity, i.e., LFY (LEAFY), AP1 (APETELA 1) and TFL1 (Ruiz-Garcia et al. 1997, Koornneef et al 1998). Several flowering time genes have been shown to affect expression of LFY, AP1 and AG. CO, GI, FVE, LD, GA1 and GAI all play a role in the activation of LFY whereas FWA, FE and FT are required for the plants to respond to LFY expression (Blasquez et al. 1998, Nilsson et al. 1998, Aukerman et al. 1999). FCA has been shown to activate both LFY and AP1 expression (Page et al. 1999). In contrast, mutations in the EMF gene lead to increased expression of AP1 and AG (AGAMOUS) (Chen et al. 1997). Similarly, TFL1 inhibits transcription of both AP1 and LFY (Ratcliffe et al. 1999).

7. Floral Induction in Oilseed Rape Species

7.1. Responses to Photoperiod and Temperature

Oilseed rape species *Brassica rapa* and *Brassica napus* are classified as annuals/spring-types or biennials/winter-types based on their flowering requirements. Flowering in annuals is influenced by photoperiod whereas biennials require vernalization (Ferreira et al. 1995, Teutonico & Osborn 1995).

The spring types are quantitative long day plants in which optimal photoperiod requirements vary between cultivars (Friend 1969, Thurling & Das 1977, King & Kondra 1986). In *Brassica campestris* (=*B. rapa*), Friend (1969) found that one long day exposure is sufficient to induce flowering and far-red light supplementation hastens the flowering response. The growing temperature can also affect flowering time. Thurling & Das (1977) reported that several cultivars of *Brassica napus* flowered earlier when grown at 15^{0}C as compared to 25^{0}C. Spring types also respond to vernalization (Thurling & Das 1977, Hodgson 1978, Murphy & Scarth 1993). The response to vernalization is quantitative, i.e., longer exposures to the cold treatment leads to decreases in flowering time. The response also varies with cultivars. Thurling & Das (1977) found that early-flowering cultivars were the least sensitive to vernalization and

under saturating light conditions, did not respond at all. The most responsive to vernalization were the late-flowering cultivars. Interestingly, the effect of vernalization in spring lines can be diminished by prior and subsequent exposures to high temperatures (30^{0}C) (Dahayanake & Galwey 1998).

The flowering response of biennial or winter-types appears to be solely controlled by vernalization (Hodgson 1978). A minimum period of vernalization is required for the flowering response to occur whereas prolonged vernalization can accelerate the flowering response (Filek & Dubert 1994). Dubert & Filek (1994) found that vernalization is most effective when plants are vernalized at the early seedling stages. Their results also showed that the optimal temperature for vernalization is higher for older seedlings. In plant vernalized 0-4 weeks after germination, no significant variations in the flowering responses were observed under vernalization temperatures of 2^{0}C and 5^{0}C. However, in plants vernalized 6-10 weeks after germination, a significantly higher percentage formed flowers when vernalized at 5^{0}C.

7.2. Biochemical Signals

GAs have been implicated in the regulation of floral induction in both spring and winter cultivars of oilseed rape species. Exogenous application of GAs to *B. napus* spring lines hastens flowering whereas application of inhibitors of GA biosynthesis results in a delay in flowering (Rood et al. 1989, Mandel et al. 1992, Dahanayake & Galwey 1999). *B. rapa* mutants that have reduced (*rosette* = *ros*) and enhanced (*elongated internode* = *ein*) GA levels have delayed and accelerated flowering, respectively (Rood et al. 1989, Zanewich et al. 1990). The *ein* mutant is also phytochrome-deficient (Devlin et al. 1992) so it is not clear if the early-flowering phenotype is a result of increased GA or due to loss of phytochrome repression of flowering. In the *B. napus* winter line Crystal, GA levels were found to increase in the shoot tips upon exposure to cold temperature (Zanewich & Rood 1995). Likewise, exogenous application of GA can induce flowering in most, but not all, biennial lines (Mandel et al. 1992).

It has been suggested that sucrose acts as a signaling molecule in floral induction in spring lines. Friend et al. (1984) were able to induce early flowering in whole *B.*

campestris plants grown in vitro by adding 40-80 mM sucrose to the culture medium. Similarly, removal of CO_2 during inductive light conditions greatly reduced flower initiation.

7.3. Genetics

Using RFLP and AFLP markers, two QTLs, designated VFN1 and VFN2, were found to exert major effects in controlling flowering time in *B. napus* spring types (Ferreira et al. 1995, Osborn et al. 1997). A third QTL, VNF3, contributes smaller effects. Similarly, the same number of QTLs was found to affect flowering time in *B. rapa* (Teutonico & Osborn 1995, Osborn et al. 1997). Again, two QTLs, named VFR1 and VFR2, account for most of the phenotypic variation in flowering time. The major QTLs in *B. napus* and *B. rapa* are collinear with *Arabidopsis* chromosome regions where several flowering time genes have been mapped (Osborn et al. 1997).

Crosses between annual and biennial cultivars indicate that the vernalization requirement is a recessive trait in *B. napus* (Van Deynze & Pauls 1994, Ferreira et al. 1995) but a dominant one in *B. rapa* (Teutonico & Osborn 1995). Van Deynze & Pauls (1994) found that vernalization requirement in *B. napus* is controlled by at least two genes, one with major effects and the other one contributing minor effects. In *B. napus* spring-types, non-responsiveness to vernalization was found to be controlled by either one gene or one QTL (Osborn et al. 1997, Murphy & Scarth 1998). In contrast, three QTLs were detected to control non-responsiveness to vernalization in *B. rapa* spring lines (Osborn et al. 1997).

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CHAPTER III

FLOWERING RESPONSES OF SPRING AND WINTER LINES OF *BRASSICA NAPUS* TO PHOTOPERIOD AND TEMPERATURE

1. Introduction

The shift from vegetative to reproductive growth is influenced by environmental conditions with light and temperature as the major controlling factors. Significant progress has been made in elucidating the genetic mechanisms by which these external cues control floral induction. This has been gained mostly from the characterization of flowering-time mutants of *Arabidopsis* (Koorneef et al. 1998, Levy & Dean 1998, Pineiro & Coupland 1998, Simpson et al. 1999). Studies on the responses of these mutants to light and cold temperature have identified three pathways regulating floral induction in *Arabidopsis*. A number of the genes involved in these pathways have been cloned and their functions have been identified. Moreover, interactions between the different mutant genes have been analyzed and as a result, an integrated model identifying the genetic mechanisms controlling floral induction in *Arabidopsis* is now available. This model, however, is still incomplete and is expected to be further refined and modified (Koornneef et al. 1998). Nonetheless, the information from this model and other genetic resources generated from *Arabidopsis* are valuable tools that can be used to facilitate research on floral induction in other plant species. It is likely that the genes involved in regulating floral induction in Arabidopsis are common to all plant species (Simpson et al. 1999). Levy & Dean (1998) have identified possible orthologues of *Arabidopsis* flowering-time genes in pea, sugar beet, barley, wheat and *Brassica* spp. However, studies on the physiology of floral induction have shown that the flowering requirements vary in different plant species (Bernier 1988, Poethig 1990, Kinet 1993). This indicates that, although a basic scheme controlling floral induction common to all plant species may exist, deviations from this scheme may have evolved in different genotypes. Therefore, in studying floral induction in other plant species using the genetic resources obtained in *Arabidopsis*, the flowering responses of these species must first be characterized.

Brassica napus lines are generally classified into two groups based on their flowering requirements; spring or annual types are quantitative long-day plants whereas winter or biennial types do not normally flower without a cold treatment. These two groups are thought to represent two distinct gene pools (Diers and Osborn 1994). The flowering responses of spring and winter lines to light and temperature have been investigated in several studies. Spring lines show a vernalization response that is both quantitative and genotype-dependent. Flowering time in spring lines decreases with increasing duration of the vernalization treatment (Murphy & Scarth 1993, Thurling & Das 1977). Murphy & Scarth (1998) evaluated the vernalization response of F1-derived DH lines derived from two crosses between three spring cultivars (Global x Karat, Karat x Marnoo) and found that the extent of response to cold treatment varied in the different DH lines. Thurling & Das (1977) identified that the early-flowering lines are the least responsive to vernalization whereas the late-flowering lines are the most responsive. Winter lines also have a similar response to vernalization. Once the vernalization requirement has been met, further vernalization can accelerate the flowering response (Filek & Dubert 1994, Tommey & Evans 1991). Dubert & Filek (1994) found that vernalization is most effective when plants are vernalized at the early seedling stages. Their results also showed that the optimal temperature for vernalization is higher for older seedlings. In plants vernalized 0-4 weeks after germination, no significant variations in the flowering responses were observed under vernalization temperatures of 2^0C and 5^0C. In plants vernalized 6-10 weeks after germination, a significantly higher percentage formed flowers when vernalized at 5^0C. These reports on the flowering responses of *B. napus*, however, do not have information on the interaction of photoperiodic induction and vernalization in winter lines.

In this study, the flowering responses of *B. napus* spring and winter lines to long-day and short-day conditions with or without vernalization were investigated. In order to determine which genes may be responsible for the obligate vernalization requirement in winter lines, genetic analysis of the spring and winter lines using RFLP markers linked to flowering time and vernalization requirement was also carried out.

2. Materials and Methods

2.1. Plant Materials

Quantum, Westar and Excel are spring cultivars. Rebel and Cascade are winter cultivars. Lines 6-200 (Vern-) and 6-202 are doubled haploid lines derived from a single Rebel by Cascade cross (Hawkins 1997). Line 6-200 lacks the vernalization requirement and Line 6-202 has retained the vernalization requirement.

2.2. Comparison of the Flowering Responses of Spring and Winter Lines to Photoperiod and Vernalization

Seeds were sown in 4-inch pots containing a soil mixture of 40% peat, 40% vermiculite and 20% coarse sand. All plants were grown in growth chambers under controlled temperatures (22/17°C day/night) and constant light intensity (400-450 uE/m^2/s) using a mixture of fluorescent and incandescent lights. Two light regimes were used; 16- hr and 8-hr photoperiods. When plants required vernalization, seedlings at the 4-leaf stage were transferred to 4°C under a 16-hrs or 8-hrs light for 6 weeks, after which they were grown at 22/17°C day/night temperatures.

The flowering responses were measured in terms of final leaf number and anthesis time (number of days until the first flower in the primary inflorescence opened). In non-vernalized plants, anthesis time was expressed as days after planting (DAP) whereas in vernalized samples, this was expressed as days after vernalization (DAV). All experiments were done three times using at least 10 samples each time. Data were analyzed by ANOVA using the GLM procedure (n=3) (SAS Institute Inc., 1999)

2.3. Assessment of Vegetative and Inflorescence Development in Westar and Line 6-200

Floral development in the primary inflorescence was studied by collecting samples at several time intervals starting from the time the seedlings germinated until the first floral bud in the inflorescence opened (anthesis). Samples from at least 10 plants were taken at each collection time. Shoot tips were collected and the leaves were carefully removed

to expose the apical meristem. The flowers were staged according to the morphological landmarks described by Smyth et al. (1990). Unless flowers showed the morphological characteristics of the stage, they were counted as belonging to that of the preceding stage. The number of expanded leaves at each collection time was also noted.

Flowers at the early stages of development were not clearly visible under the dissecting microscope. In these stages, the inflorescences were processed for scanning electron microscopy (SEM) according to the procedure described by Smyth et al. (1990) with a few modifications. Samples were fixed in 3% gluraraldehyde in 0.025M sodium phosphate buffer (pH 6.8) at 4°C for at least 12 hrs, after which they were rinsed twice with the buffer. They were then further fixed in 1% osmium tetroxide in 0.025M sodium phosphate buffer (pH 6.8) at room temperature for 4 hrs or until the osmium tetroxide turned black. The samples were then washed with distilled water (4 changes) for two hrs. Samples were dehydrated through an alcohol series (10-100% ethanol at 10% intervals; 30 mins in each alcohol concentration). Samples were stored at 100% ethanol until they could be examined by SEM. Prior to critical point drying, the samples were run through a dehydration/intermediate fluid series (30 min each in ethanol:amyl acetate solutions; 3:1 parts, 2:2 parts, 1:3 parts) and finally an intermediate fluid rinse using 100% amyl acetate. Samples were then brought to the SEM Lab, Dept. of Earth and Atmospheric Sciences, Univ. of Alberta for microscopy.

2.4. Genomic DNA Extraction and Restriction

Genomic DNA extraction was performed according to Dellaporta et al. (1983) with a few modifications, i.e., Proteinase K was removed from the extraction buffer and RNAse treatment was done prior to nucleic acid purification. Approximately 5 gms of young leaves was ground in liquid nitrogen using a mortar and pestle. The liquid nitrogen was allowed to evaporate and 8 mls of the extraction buffer (100 mM Tris pH 8, 50 mM EDTA, 500 mM NaCl, 10 mM mercaptoethanol) was added to the tissue The sample was ground further and then transferred to a 30-ml tube. One ml of 20% SDS was added and the slurry was mixed thoroughly by vortexing. The sample was then incubated at 55°C for I hr, after which, 5 mls of 5M potassium acetate was added and the sample was mixed by gently inverting the tube several times. It was then placed on ice for 20 mins. The sample was centrifuged at 15,000 rpm for 10 min and the

supernatant was filtered through a single layer of Miracloth (Calbiochem). To remove contaminating RNAs, RNAse was then added to a final concentration of 10 µg/ml and the sample was incubated at 37°C for I hr. An equal volume of phenol/chloroform (25:24:1 phenol:chloroform:isoamyl alcohol) was then added to the sample. It was centrifuged for 10 mins at 15,000 rpm and the aqueous phase was collected. An equal volume of chloroform (24:1 chloroform:isoamyl alcohol) was added and the sample was again centrifuged. The aqueous layer was collected and DNA was precipitated by the addition of 0.6 vol of isopropanol and placing the sample on ice for at least 30 mins. DNA was collected by centrifugation at 5,000 rpm for 10 mins. The DNA was rinsed twice with 70% ethanol, dried under vacuum and resuspended in 100 µL TE (10 mM Tris pH8, 1mM EDTA) to avoid DNA degradation during prolonged storage.

DNA quality and concentration was determined by visualizing ethidium bromide-stained bands using a UV transilluminator. Five µL of the DNA sample was loaded onto 1% SeaKem agarose gel (FMC Bioproducts) with 0.5 ng/ml ethidium bromide and electrophoresed for 1 hr at 100 V. DNA band intensity was compared with known concentrations of λDNA. Approximately 10 µg of DNA was digested with 50 U of Hind III or EcoR1 (Gibco-BRL) at 37°C overnight.

2.5. Southern Blotting

Ten µg of digested DNA samples were loaded onto 1% SeaKem agarose gel, electrophoresed for 3-4 hrs at 100 V and then blotted onto Zeta-Probe Membrane (Bio-Rad Laboratories) using the alkaline transfer method specified by the manufacturer. Probes were labelled using the Multi-Prime DNA Labelling Kit (Amersham). Hybridization (formamide method) and high stringency washes were done according to the protocol described by the membrane manufacturer (Bio-Rad Laboratories).

2.6. RFLP Markers Used as Probes

RFLP markers were obtained from Dr. Tom Osborn (Dept. of Agronomy, Univ. of Wisconsin). Five markers belonging to LG9 (WG7F3A, WG6B10, WG8G1b, WG5A5, TG6A12a) and 3 markers in LG12 (EC3G3C, WG7B3, WG1G4) were used as probes.

These markers were found to be strongly linked to flowering time in a segregating population derived from a *B. napus* Stellar (spring) x Major (winter) cross (Ferreira et al. 1995, Osborn et al. 1997). Hawkins (pers comm) previously evaluated the LG 9 markers in an F2 population derived from a *B. napus* 6-200 (spring) x Cascade (winter) cross. A strong association between these markers and flowering time was similarly detected with the exception of WG5A5.

3. Results

3.1. Flowering Responses of Spring and Winter Lines to Photoperiod and Vernalization

Table 1 and 2 shows the mean leaf number and anthesis time of the different *B. napus* lines grown under long- and short-day growth conditions, with or without vernalization. Leaf number indicates the developmental time at which the plant shifts from vegetative to reproductive growth. Anthesis time measures the duration of both floral induction and floral morphogenesis phases.

The spring lines Quantum, Excel, Westar and 6-200 were both photoperiodic and vernalization responsive, with the vernalization response more evident when the plants were grown under limiting light conditions. Photoperiod is a major controlling factor for the spring lines even when they are vernalized. Under the 8-hr photoperiod, flowering times, both in terms of leaf number and actual anthesis time, were significantly delayed. Although vernalization could not totally compensate for the photoperiod requirement (leaf number was significantly higher in plants grown under 8-hr light/cold treatment compared to those under 16-hr light), it could hasten flowering under the limiting light conditions. Further, the effects of vernalization and light were additive in Line 6-200 and Excel as indicated by the significant decrease in leaf number in vernalized plants grown under long day conditions. This, however, was not observed in Quantum and Westar. It is possible that 16-hr light is already the optimal photoperiod in these lines and thus, longer daylength or vernalization does not further reduce leaf number or anthesis time. This response has been previously noted (King & Kondra 1986, Martinez-Zapater et al. 1994). Generally, vernalization combined with long day photoperiod led to the shortest flowering times in terms of leaf number in the spring lines.

Table 1. Mean final leaf number of B. napus lines under 16- and 8-hr photoperiods with or without vernalization

Plant Lines	Without Vernalization (DAP)		With Vernalization (DAV)	
	16-hr	8-hr	16-hr	8-hr
Quantum	15.2 a	33.6 e	14.7a	21.4j
Westar	14.2 ah	26.8 d	13.3 h	18.6 i
6-200	16.5 b	23.5 f	14.7 a	17.9 i
Excel	17.4 bi	29.4 g	13.4h	20.8 cj
Rebel Early	19.9 c	DNF	20.1c	24.0 f
Rebel Late	26.9 d	DNF	18.9i	24.3 f
Cascade	DNF	DNF	18.1 i	17.2 bi
6-202	DNF	DNF	17.4 bi	17.8 l

*Values followed by the same letter are not significantly different at $p<0.05$
DNF = did not flower
DAP = days after planting
DAV = days after vernalization

Table 2. Mean anthesis time of B. napus lines under 16- and 8-hr photoperiods with or without vernalization

Plant Lines	Without Vernalization (DAP)		With Vernalization (DAV)	
	16-hour	8-hr	16-hr	8-hr
Quantum	44.3 a	150.4 e	14.6 i	57.1m
Westar	50.0 bc	126.4 f	21.4 j	52.1 c
6-200	52.2 c	107.1 g	25.2 k	49.5 c
Excel	46.5 ab	121.6 h	14.5 i	42.5 a
Rebel Early	55.2 c	DNF	22.4 j	65.9 d
Rebel Late	68.3 d	DNF	20.9j	63.7 d
Cascade	DNF	DNF	20.6 j	46.8 ac
6-202	DNF	DNF	28.7 l	66.8 d

*Values followed by the same letter are not significantly different at $p<0.05$
DNF = did not flower
DAP = days after planting
DAV = days after vernalization

Flowering of the doubled haploid line 6-200 was not as sensitive to photoperiod as compared to the other spring cultivars, Quantum, Excel and Westar. Comparing non-vernalized plants grown under 8-hr and 16-hr photoperiods, the increase in leaf number (42%) and anthesis time (105%) in Line 6-200 under SD is shorter relative to the other spring-types (69-121% in leaf number, 153-240% in anthesis time). Moreover, vernalization greatly diminished the delaying effect of SD in Line 6-200. Leaf number in vernalized Line 6-200 plants grown in SD increased by just 8% as compared to non-vernalized plants grown in LD. In the other spring lines, the increase was 19-40%.

The winter cultivar, Rebel, was found to lack a vernalization requirement. Furthermore, the breeder seed segregated into early- and late-flowering phenotypes under LD. Thus, after single seed descent to homozygosity, the flowering responses of the two were determined. The two did not flower under SD without vernalization. When vernalized, both have delayed flowering under SD as compared to LD. The late-flowering Rebel was more responsive to vernalization as compared to the early-flowering line.

The obligate winter types, Cascade and Line 6-202, did not flower without cold treatment. Moreover, when vernalized, they produced similar leaf numbers when grown under 16- or 8-hrs of light. Anthesis was delayed in Cascade and Line 6-202 when grown under 8-hr photoperiod by 127% and 133%, respectively.

A major difference in the response of spring and winter lines to photoperiod can be clearly seen when correlation analysis is done between changes in leaf number and anthesis time under LD and SD conditions (Table 3). In the spring lines, there is high correlation between leaf number and flowering time under vernalizing or non-vernalizing conditions, i.e, an increase in leaf number is reflected with a delay in flowering time. This was not seen in Line 6-202 or Cascade. This indicates that, in these winter lines, photoperiod does not influence the developmental time at which the plants shift to reproductive growth. The delay in anthesis time seen under the limiting light condition does not reflect a delay in floral induction but rather, is due to the overall slower growth of the plants.

Table 3. Correlation analysis between leaf number and flowering time under vernalized and non-vernalized conditions

Plant Line	R Square Value	
	Without Vernalization	With Vernalization
Quantum	0.9972	0.9863
Excel	0.9986	0.9739
6-200	0.9939	0.8520
Westar	0.9990	0.9699
Rebel E	NA	0.9952
RebelL	NA	0.9802
6-202	NA	0.7095
Cascade	NA	0.5690

NA = not applicable

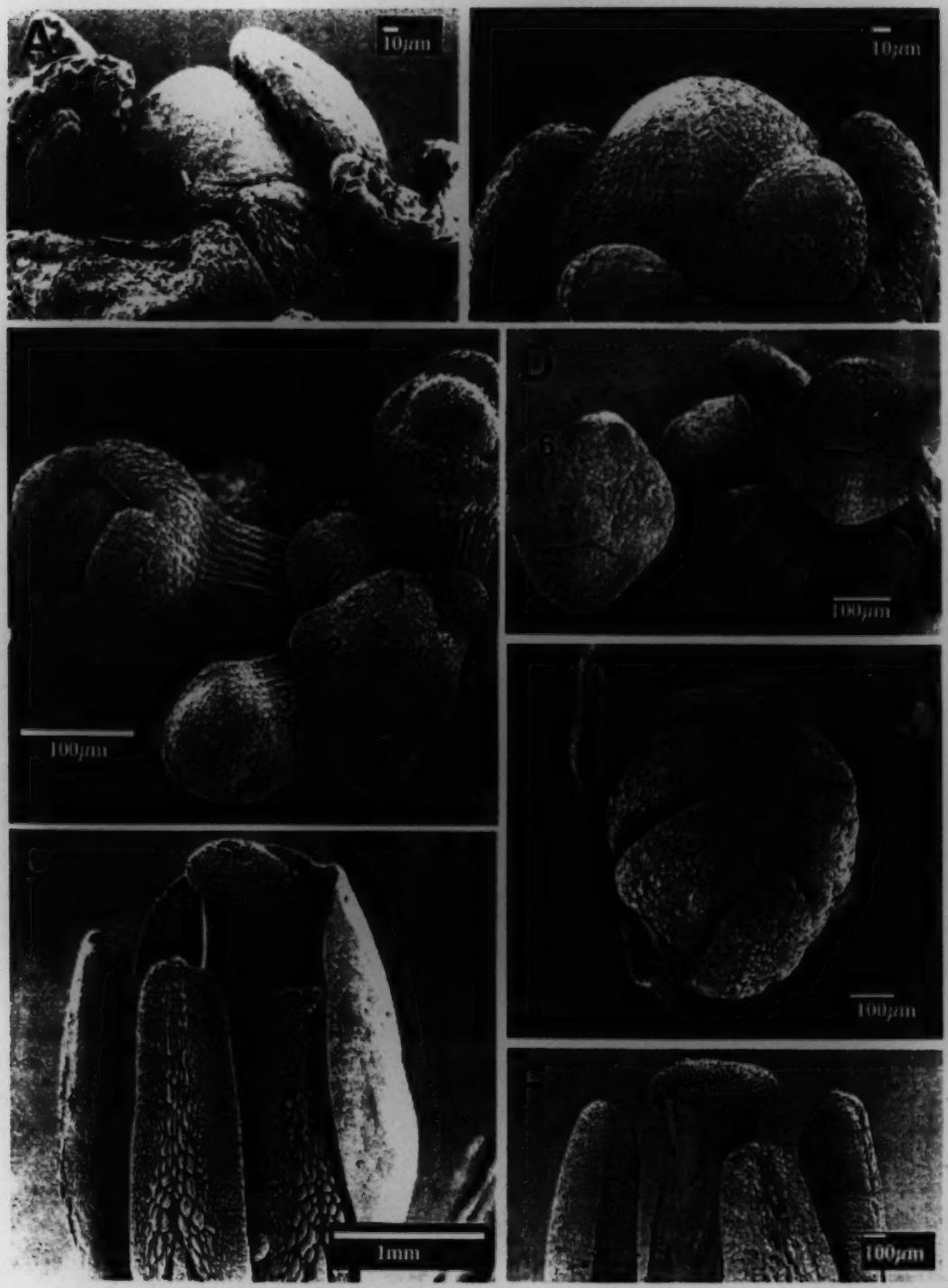
3.2. Vegetative and Inflorescence Development in Westar and Line 6-200

It was noted in the flowering responses of the spring lines that higher final leaf number, which indicates late transition to reproductive growth, does not necessarily translate to longer anthesis time. In *Arabidopsis*, final leaf number is the preferred measure to indicate transition to reproductive growth since anthesis time also indicates the duration of inflorescenceand floral development which may vary between different ecotypes (Martinez-Zapater et al. 1994). Westar produced fewer leaves compared to 6-200 but the two had the same anthesis time (Table 1 and 2). To determine if this is due to differences in the duration of floral morphogenesis, vegetative and inflorescence development in Westar and 6-200 were studied.

Smyth et al. (1990) described 12 morphological landmarks dividing floral development in *Arabidopsis* into 12 stages. This can also be used in *B. napus* with the exception of Stage 10 which commences when the petals are level with the short stamens. This stage was not very distinct in *B. napus* wherein all the stamens are of equal sizes but two have lower insertion sites making them appear shorter. Therefore, based on the classification described for *Arabidopsis*, floral development in *B. napus* can be divided into 11 stages. In this study, only 7 of these stages were scored based on the ease with which they can be observed under light and scanning electron microscopy. These stages are shown in Figure 2.

Grown under 16-hr photoperiod, Westar and Line 6-200 plants were collected at different time intervals and checked for leaf number and presence of vegetative or inflorescence meristem. At 14-DAP, both Westar and Line 6-200 were at the 2-leaf stage and were still in the vegetative phase of growth. At 18-DAP, almost half of the seedlings had shifted to reproductive growth (Westar = 53%, Line 6-200 = 47%) and by 20-DAP, most seedlings were in the reproductive phase (Westar = 94%, Line 6-200 = 95%). Line 6-200 passed through the vegetative phase faster than did Westar (Figure 3), initiating more leaves in that time frame. Figure 4 shows the timing of inflorescence development in Westar and Line 6-200. Both shifted to reproductive growth at the same time. Inflorescence development also proceeded at approximately the same rate. The appearance of Stage 8 flowers in Line 6-200 and Westar differed only by at most one

Figure 2. Inflorescence development in *B. napus*. (A) vegetative meristem; (B) inflorescence meristem; (C) inflorescence showing early stages of floral morphogenesis: Stage 1 - appearance of floral buttress in the inflorescence meristem, Stage 2 - formation of a definite floral primordium, Stage 3 - formation of sepal primordia and Stage 4 - sepals begin to overlie the floral meristem; (D) Stage 6 - sepals cover the floral meristem; (E) Stage 8 - appearance of locules in the stamens, (F) Stage 10 - formation of stigmatic papillae; (G) Stage 11 - petals cover the stamens.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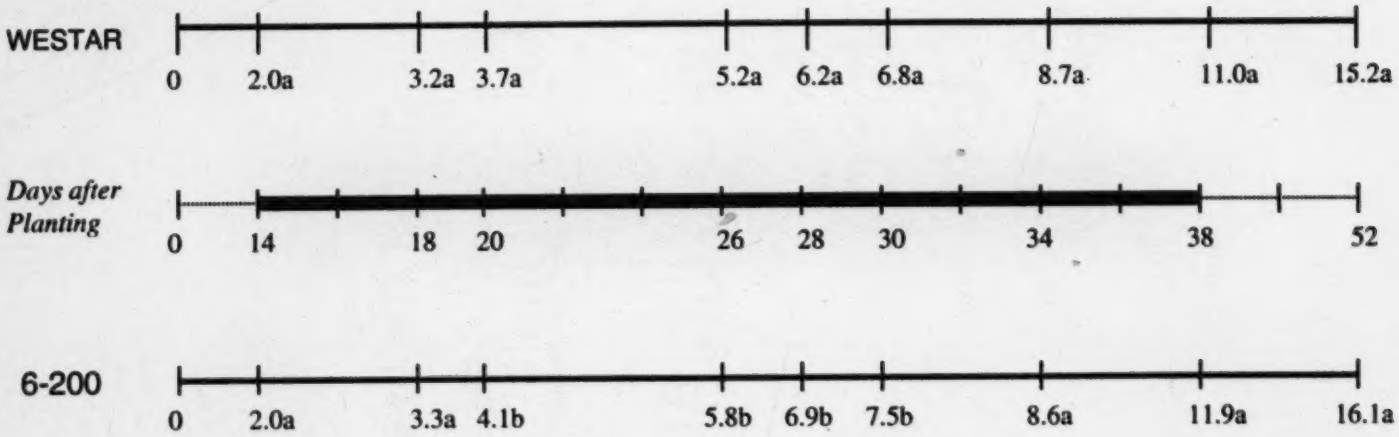
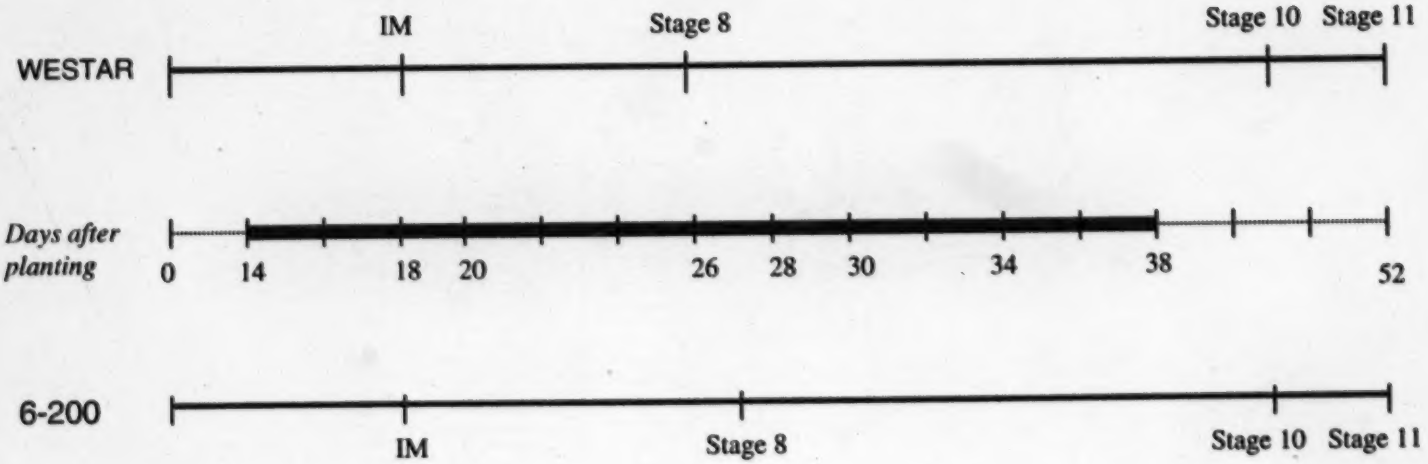
100μm

Figure 3. Vegetative development in Westar and Line 6-200.



*Values represent mean number of leaves at each sampling date. Means at each sampling date were compared by one-way ANOVA at $p>0.05$ ($n = 10$). Westar and Vern- values followed by the same letter are not significantly different.

Figure 4. Inflorescence development in Westar and Line 6-200.



day. Eventually, Stages 10 and 11 flowers appeared in Westar and Line 6-200 at the same time appeared in Westar and Line 6-200 at the same time. As a result, the flowering time of Line 6-200 was not significantly different as to that of Westar. Line 6-200 and Westar had the same flowering time although Line 6-200 produced more leaves due to rapid vegetative growth.

3.3. RFLP Analysis in 6-202, Rebel and Cascade

In order to determine which genes may be controlling the obligate vernalization requirement in winter lines. RFLP analysis was done in the early- and late-flowering Rebel lines, 6-202 and Cascade using markers linked with the QTLs VFN1 (LG9) and VFN2 (LG12). These QTLs were found to be collinear with several flowering time genes in *Arabidopsis* (Osborn et al. 1997). RFLP analysis of 6-200 using these markers have been reported earlier (Hawkins et al. submitted for publication)

Of the 8 RFLP markers tested, polymorphisms between the early- and late-flowering Rebel lines were detected using the LG9 RFLP markers WG6B10 and WG7F3A (Figure 5A and 5B). In comparison, the late-flowering Rebel, Line 6-202 and Cascade had similar RFLP patterns using WG6B10 whereas using WG7F3A, the late-flowering Rebel and Line 6-202 showed no polymorphism. Using the LG12 markers WG7B3 and EC3G3C, no polymorphisms were observed between the early- and late-flowering Rebel (Figure 6A and 6B). Similarly, Line 6-202 and Cascade had the same RFLP patterns using these markers. However, polymorphism between these two groups of vernalization-requiring (Line 6-202 and Cascade) and non-requiring (early- and late-flowering Rebel) lines were detected using these LG12 markers. Based on these results, the obligate vernalization requirement in Line 6-202 and Cascade appears to be controlled by loci in LG12 whereas flowering time differences in the early- and late-flowering Rebel are probably due to loci in LG9.

Figure 5. RFLP analysis of Rebel, Line 6-202 and Cascade using WG6B10 (A) and WG7F3A (B). Genomic DNA was digested with Hind III.

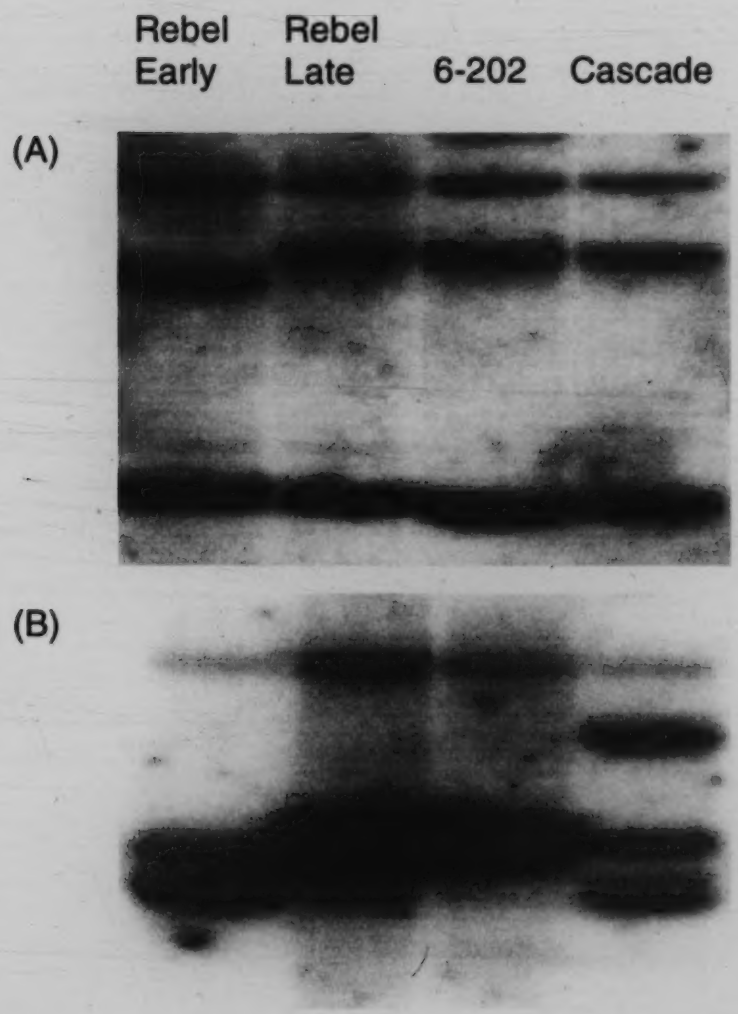
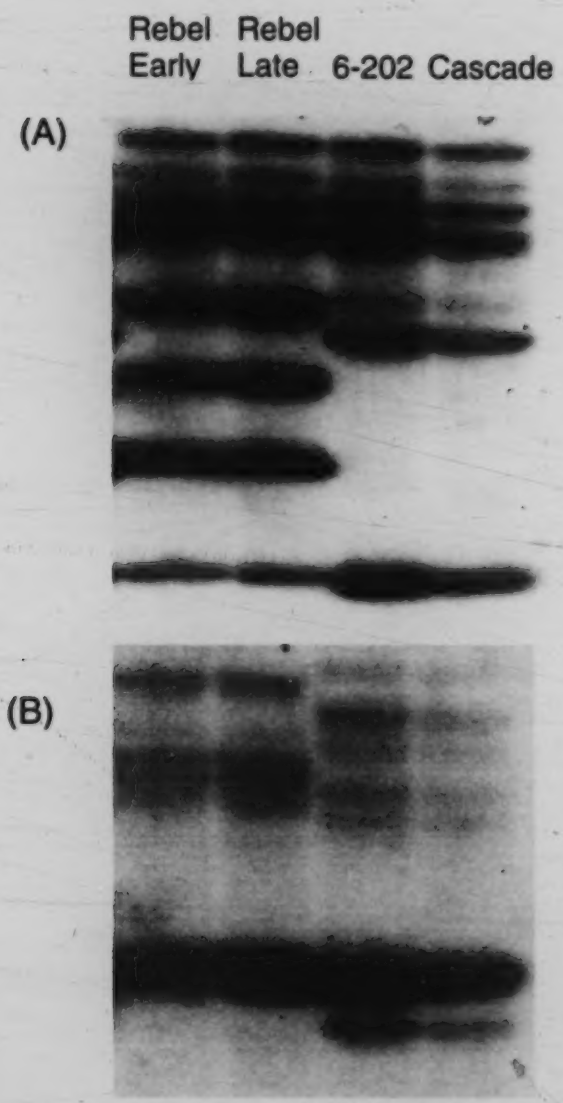


Figure 6. RFLP analysis of Rebel, Line 6-202 and Cascade using EC3G3Ca (A) and WG7B3 (B). Genomic DNA was digested with Hind III.



4. Discussion

The spring *B. napus* lines used in this study were both photoperiodic and vernalization responsive, with light as the main factor regulating the transition into reproductive growth. This primary role of light is shown by: (1) the significant delay in floral induction seen in vernalized plants grown under SD compared to non-vernalized plants grown under LD, and (2) the response to vernalization is more evident when plants are grown under SD. Previous studies on other *B. napus* spring lines have shown that they always respond to vernalization (Thurling & Das 1977, Hodgson 1978, Murphy & Scarth 1993). As such, the vernalization pathway promoting floral induction seems to be present in all spring genotypes. Murphy & Scarth (1998) reported that the spring line Karat and several DH lines derived from spring crosses with Karat are not vernalization responsive. This, however, does not reflect the absence of the vernalization pathway in these lines since the plants were grown under LD. Instead, as shown in Quantum and Westar, this indicates lower optimal photoperiod requirements. Thurling & Das (1977) found that *B. napus* spring lines that had been vernalized for 6 wks had the same flowering time when grown under 12-hr or continuous light. They then concluded that fully vernalized plants no longer show photoperiodic response and thus, vernalization can compensate for SD conditions. In this study, a 6-wk vernalization period did not compensate for the delay in flowering in plants grown under 8-hr photoperiod. This shows that a "fully-vernalized" state is dependent upon the light regime; the shorter the photoperiod, the longer vernalization period is required to eliminate the photoperiodic response. This observation further supports the primary role of light in regulating flowering in spring lines.

In *Arabidopsis*, a third pathway termed constitutive or autonoumous, is present and it is responsible for floral induction in the absence of inductive LD or vernalization treatments (Koornneef et al. 1998, Levy & Dean 1988). The *B. napus* spring lines were able to flower under 8-hr photoperiod and thus, the constitutive pathway is also found in this species.

The early- and late-flowering Rebel did not flower at all under SD. Rebel was originally classified as winter type. Mandel et al. (1992) reported another winter cultivar WW1033 that also flowered without vernalization when grown under continuous light. As in Rebel, WW1033 is highly photoperiodic wherein it will not flower when the cycle has less than 17 hours of light. Moreover, vernalized WW1033 plants require LD for flowering (Mandel 1977). WW1033 and the original Rebel seeds were probably extremely late-flowering lines and as a result, they have been classified as winter types. The original Rebel breeder seed was reported to have a flowering time of 84 days under a 16-hr photoperiod (Hawkins et al., submitted for publication).

In the winter lines used in this study, floral induction is promoted solely by vernalization. Cascade and Line 6-202 did not flower under LD without cold treatment. Moreover, once vernalized, photoperiod did not influence the developmental time at which these lines shifted to reproductive growth. These results show that these winter lines are not able to respond to long day promotion of flowering and thus, this pathway is probably non-functional in these lines. Therefore, the obligate vernalization requirement in these winter lines is a result of defects in this pathway. RFLP analysis done in Rebel, 6-202 and Cascade indicates that the obligate vernalization requirement is controlled by loci in LG12. LG12 was found to be collinear with *Arabidopsis* chromosome 5 where the flowering time genes CO, FY, EMF1 and FLC are located (Osborn et al. 1997). FY is a gene involved in the promotion of flowering in the constitutive pathway (Levy & Dean 1998). CO (CONSTANS) is a gene involved in the long day promotion of floral induction (Simpson et al. 1999). Mutation in CO results in late flowering under LD conditions (Koornneef et al. 1991). The fact that mutation in CO does not lead to a totally non-flowering phenotype indicates that CO alone may not completely account for the obligate vernalization requirement in *B. napus* winter lines. As Line 6-202 and Cascade failed to flower under both SD and LD, the obligate vernalization requirement may be due to defects in both the photoperiodic induction and constitutive pathways. Both CO and FY may be non-functional in the winter lines. This is consistent with the winter habit being a recessive trait, i.e., the obligate vernalization requirement is the result of absence of gene product(s).

Line 6-200 is an early-flowering spring line developed from a cross between Rebel and Cascade. Compared to the other spring lines, Line 6-200 is less sensitive to

photoperiod and this is probably due to its winter genetic background. Hawkins (1997) reported that the field performance of Line 6-200 in terms of maturity exceeds that of the other spring checks and this is due to rapid vegetative and reproductive phases in this line. Under controlled growth conditions, the shift to reproductive growth and floral development in Line 6-200 and Westar proceeded at approximately the same time. Vegetative growth, though, was more rapid in Line 6-200. This trait, however, cannot be claimed to be uniquely characteristic of Line 6-200 since Excel also produced more leaves and had relatively shorter flowering time. It was noted in this study that *B. napus* spring lines that have more leaves do not necessarily have longer anthesis time due to rapid vegetative growth. Hence, "early-flowering" in terms of leaf number does not necessarily translate to "early-flowering" in terms of actual time. Therefore, it cannot be used as a measure of "earliness" for plant breeding purposes. However, the importance of determining leaf number should not be understated. Taken together with anthesis or flowering time, the contribution of the vegetative growth rate to flowering time can be taken into account. Therefore, in breeding early-flowering lines, one cannot only manipulate genes involved in floral induction but one can also look at the genetic factors controlling rate of vegetative growth.

It has been shown in this study that the inability of Cascade and Line 6-202 to respond to light-mediated induction of flowering accounts for their obligate vernalization requirement. The next question now is: what are the gene or genes involved in regulating the vernalization response in these *B. napus* winter lines? Based on the RFLP analysis, this could either be EMF1 or FLC. The EMF1 and FLC genes are thought to have central roles in floral induction in *Arabidopsis* (Haughn et al. 1995, Koornneef et al. 1998, Sheldon et al. 2000). While this function in EMF1 has only been deduced from genetic interactions with other flowering time genes (Yang et al. 1995), this has been shown in FLC based on expression studies (Michaels & Amasino 1999; Sheldon et al. 1999, 2000). FLC encodes a repressor of flowering that is down regulated by vernalization. Levels of FLC transcript have been directly correlated with leaf number/flowering time of early- and late-flowering ecotypes and in the vernalization response of cold treated plants. FLC is, therefore, the gene most likely to be involved in regulating the vernalization response in these winter lines.

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CHAPTER IV

EXPRESSION OF THE FLC (FLOWERING LOCUS C) GENE IN SPRING AND WINTER LINES OF *BRASSICA NAPUS*

1. Introduction

Vernalization is the acquisition or the acceleration of the ability to flower by exposures to chilling temperatures (Chouard 1960). In many temperate species, vernalization is an adaptive mechanism to ensure that flowering occurs in the spring when environmental conditions are favourable for growth, floral and seed development (Dennis et al. 1996). While this mechanism is advantageous to the plants, from the perspective of plant breeders, it impedes the use winter or biennial lines to expand the genetic resources in spring or annual lines and to exploit heterotic effects in hybrid crosses. In oilseed rape species, for example, yields from winter lines far exceed yields obtained from spring lines (Canola Growers Manual 1991). Moreover, Butruille et al. (1999) reported that hybrids from crosses between winter and annual *B. napus* lines have consistently higher yields compared to hybrids resulting from two spring parents. Therefore, understanding the genetic basis of vernalization requirement is of importance in crop improvement.

In *Arabidopsis*, the genetic basis of vernalization requirement has been studied in ecotypes that are late flowering under long day conditions but are early flowering when exposed to cold treatments. In these ecotypes, the late flowering phenotype is conferred by the synergistic actions of two genes, FRI and FLC, whose effects are overcome by vernalization (Lee et al. 1993, Clarke & Dean 1994, Lee et al. 1994, Lee & Amasino 1995). The late alleles of FRI are dominant whereas the late FLC alleles are semidominant in their action (Michaels & Amasino 1995). The presence of both late FRI and FLC alleles results in extremely late flowering whereas a combination of late and early alleles in these loci leads to relatively early flowering. The earliest flowering phenotype is observed when both loci have the early alleles. Studies on the genetic interactions of FLC with several flowering time mutants in genes found in the

photoperiodic induction and autonomous pathways have indicated that FLC may have a central role in floral induction (Sanda & Amasino 1996).

The identity of the FRI gene is still unknown. On the other hand, the FLC gene has been isolated, sequenced and identified as a MADS-domain type of transcription factor which represses flowering (Michaels & Amasino 1999, Sheldon et al. 1999). It is highly expressed in late-flowering ecotypes and its expression is down regulated by vernalization. It is unaffected by photoperiodic conditions. The levels of FLC transcript and protein are directly correlated with the duration of cold treatment and the resulting leaf number/flowering time (Sheldon et al. 2000). Moreover, FLC expression is increased in late-flowering vernalization responsive mutants but not in late-flowering vernalization non-responsive mutants (Sheldon et al. 1999). Taken together, these results show that FLC is involved in directly controlling the vernalization response and is the central regulator of flowering in the vernalization pathway.

Studies on vernalization in *Arabidopsis* were carried out using late-flowering ecotypes that still flower without cold treatment. Hence, it is uncertain if the information from these studies also holds true in genotypes wherein there is an obligate vernalization requirement. These late-flowering ecotypes in *Arabidopsis* are essentially the equivalent of late-flowering spring lines in *B. napus*. In this study, FLC expression was checked in spring and winter lines of *B. napus*. Further, FLC expression was checked in the winter line 6-202 that has been vernalized under different conditions.

2. Materials and Methods

2.1. Growth Conditions

Seeds were sown in trays or 4-inch pots containing a soil mixture of 40% peat, 40% vermiculite and 20% coarse sand. All plants were grown in the greenhouse under 20°C day/night temperatures and 16-hrs light supplemented by 400 W sodium vapor lamps (HID Sylvania Canada) to maintain a minimum light intensity 275-300 $\mu mol/m^2/s$. Vernalization was done in growth chambers at 4°C under a 16-hr photoperiod using a mixture of fluorescent and incandescent lights.

2.2. Sample Collection

All experiments on FLC expression were carried out at least twice.

2.2.1. Non-vernalized Spring and Winter Lines

The spring lines used for this part of the study were Quantum, Excel, Westar, 6-200, Rebel-early, Rebel-late in addition to two obligate winter lines, 6-202 and Cascade. Plants were grown until the 4-leaf stage and then shoot tips, leaves and roots were collected. Shoot tips were collected from 25-30 plants while leaf and root samples were taken from 5 plants. For leaf samples, the 2 youngest expanded leaves (3rd and 4th leaves) were harvested.

2.2.2. Vernalized Line 6-202

Preliminary experiments were done to check if FLC expression in Line 6-202 was down regulated by cold treatment and the corresponding decrease in transcript level was correlated with leaf number and flowering time. Line 6-202 plants were grown to the 4-leaf stage, and then vernalized for 1 or 6 wks. At the end of the vernalization periods, shoot tips and leaf samples (two youngest expanded leaves) were collected.

Line 6-202 was vernalized at the seed, cotyledonary and 4-leaf stages for 4, 5 and 6 wks. Seeds were allowed to imbibe water overnight prior to transfer to the vernalizing conditions. In plants vernalized at the seed and cotyledonary stages, shoot tips, leaves (or cotyledons) and roots were collected at the end of the vernalization period and when the plants had reached 2- and 4-leaf stages. In plants vernalized at the 4-leaf stage, samples were collected at the end of the vernalization period and 24 hrs after the plants have been moved to the greenhouse. It was observed earlier that Line 6-202 plants vernalized at the 4-leaf stage for 6-wks still had vegetative meristem at the end of the vernalization period but shifted to reproductive growth within 24 hrs of transfer to greenhouse conditions. Tissues were also collected from control non-vernalized plants.

Each time, shoot tips were collected from 25-30 plants while leaf and root samples were taken from 5 plants. Root growth in plants vernalized prior to seed germination

and collected at the end of the vernalization period was not very extensive so samples from 40-50 plants were collected to extract enough RNA. For leaf samples taken at different seedling stages, the two youngest expanded leaves (1st and 2nd leaves at the 2-leaf stage, 3rd and 4th leaves at the 4-leaf stage) were harvested.

The flowering response of Line 6-202, as influenced by duration of vernalization treatment and seedling age at which the cold treatment is given, was also evaluated. The flowering responses were measured in terms of final leaf number and flowering time (DAV = days after vernalization). All experiments were done three times using at least 10 samples each time. Data were analyzed by ANOVA using the GLM procedure (n=3) (SAS Institute Inc., 1999)

2.3. Total RNA Extraction

Total RNA was extracted according to the procedure described by Verwoerd et al. (1989) with a few modifications, i.e., volumes of extraction buffer and water were increased. The extraction buffer (1:1 phenol: 0.1M LiCl, 100mM Tris pH8, 10mM EDTA, 1% SDS) was kept at 80°C. The samples were ground in liquid nitrogen and transferred to appropriate tubes. Approximately 500 µl (for shoot tips and cotyledons) or 2 ml (for leaves and roots) of the hot extraction buffer were added to each sample. The mixtures were homogenized by vortexing for at least 30 secs. Equal volumes of chrloroform:iso-amyl alcohol (24:1) were then added and the samples were again mixed by vortexing. The samples were centrifuged (13,000 rpm for 15 mins at 4°C), the aqueous phase collected and an equal volume of 4M LiCl was added. The RNAs were allowed to precipitate overnight at 4°C overnight. RNAs were collected by centrifugation and the pellets were dissolved in 300 µl water. The RNA samples were further purified by precipitation with 0.1 volume of 5M NaCl and 2 volumes of 100% ethanol. RNAs were then washed twice with 70% ethanol, dried under vacuum and resuspended in 20 µl (shoot tips, cotyledons), 50 µl (roots) or 100 µl (leaves) of water. RNA concentrations were quantified using GeneQuant (Pharmacia-LKB). Samples were stored in -80°C until further use.

2.4. Northern Blotting

Approximately 20 µg (shoot tips) or 30 µg (cotyledons, leaves, roots) of RNA samples were loaded in 1% denaturing agarose gel (Sambrook et al. 1989) and electrophoresed for 2-3 hrs at 100 V. RNA was transferred to Zeta-Probe Membrane (Bio-Rad Laboratories) using the method specified by the manufacturer. Probes were labelled using the Multi-Prime DNA Labelling Kit (Amersham). Hybridization (standard protocol) and high stringency washes were completed according to the procedure described by the membrane manufacturer (Bio-Rad Laboratories).

2.5. FLC Probe

The FLC probe was obtained from Dr. Rick Amasino (Dept. of Biochemistry, Univ. of Wisconsin). It was a 0.9 Kb fragment of the FLC gene of *B. rapa* without the MADS-box sequences (Amasino, pers comm).

3. Results

In tissues of all lines examined, FLC expression was consistently lower in the spring lines (Figure 7). This differences in FLC expression was most evident in the shoot tips where the shoot apical meristem is found, the site of perception of cold signal (Metzger 1988) and the organ which will eventually give rise to the floral structures.

Initial experiments were done to determine if FLC expression is down regulated by cold and the resulting transcript levels correlates with flowering time and leaf number. Line 6-202 failed to flower when grown to 4-leaf stage and then vernalized for 1 wk. Extending the vernalization period to 6 wks resulted to 100% flowering with an average flowering time of 33 days and final leaf number of 16. FLC levels in these plants were checked at the end of the vernalization periods (Figure 8). Levels of FLC transcripts in shoot tips and leaves were down regulated by the cold treatment. FLC expression was higher in plants vernalized for 1 wk compared to those exposed for 6 wks. Also, FLC levels in plants vernalized for 1 wk was considerably lower when compared to plants that had not been vernalized. This down regulation of FLC level by 1 wk exposure to cold

Figure 7. FLC expression in shoot tips (A), leaves (B) and roots (C) of non-vernalized spring and winter lines of *B. napus*. Q – Quantum; E- Excel; W – Westar; RE – Rebel Early; RL – Rebel Late, C - Cascade

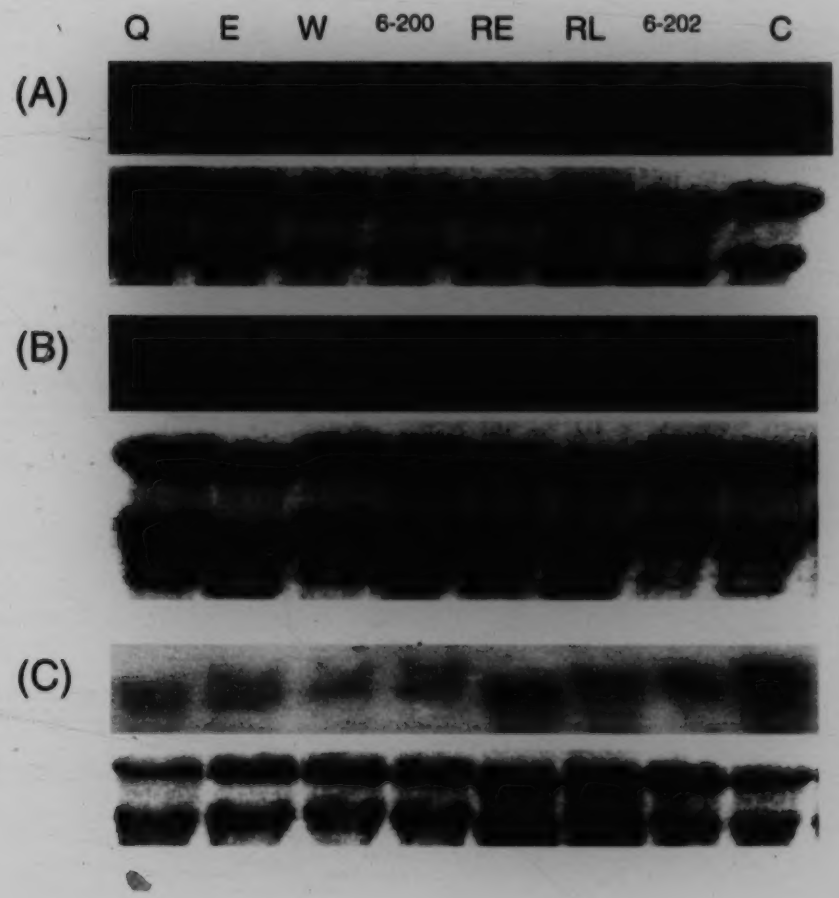
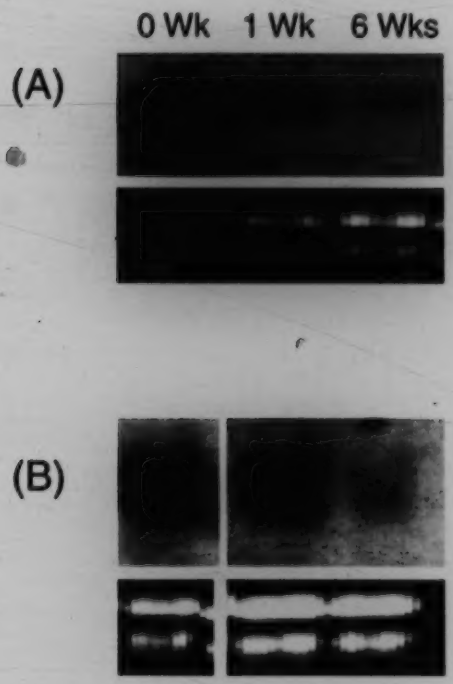


Figure 8. FLC expression in shoot tips (A) and leaves (B) of Line 6-202 vernalized for 0, 1 or 6 weeks.



treatment, however, did not result in flowering.

Aside from duration of vernalization period, the vernalization response of winter lines is also affected by the developmental age at which the cold treatment is given to the plants (Dubert & Filek 1994). In this study, it was further demonstrated that the quantitative response to length of vernalization period is influenced by the developmental age at which the cold stimulus is given to the plant (Table 4). When Line 6-202 was vernalized at the seed and 4-leaf stages, no significant differences were observed in the flowering responses of plants exposed to the cold treatment for 4-6 wks. In comparison, when Line 6-202 was vernalized at the cotyledon stage, leaf number was significantly higher in plants vernalized for 4 wks as compared to those that have been in the cold conditions for 6 wks. Apparently, the optimal vernalization period for plant vernalized at the early stages of vegetative growth is longer as compared to seed or older seedlings. Moreover, plants vernalized at the 4-leaf stage for 4 wks had less leaves as compared to plants vernalized at the cotyledonary stage for the same duration. This is despite of the fact that plants at the 4-leaf stage would have produced more leaves before transfer to the vernalizing conditions.

FLC expression was checked under these conditions. In control non-vernalized plants, FLC expression in the shoot tips, leaves/cotyledons and roots increased as the plant aged (Figures 9, 10 & 11). During the cold treatment, FLC expression was down regulated as shown by the decrease in transcript levels seen in samples collected at the end of the vernalization period. No differences in transcript levels were seen under the various vernalization conditions. This is consistent with the flowering response of plants vernalized at the seed and 4-leaf stages. FLC expression, however, did not reflect the delay in floral induction seen in plants vernalized at the cotyledonary stage for 4 wks. What is even more surprising was that FLC expression progressively increased when the vernalized plants were transferred to the normal growing temperatures. FLC expression in all the tissues increased, albeit more evident in shoot tips, even when the plants had already shifted into the reproductive stage. In plants vernalized at the seed and cotyledonary stages for 6 weeks, the plants had already formed floral structures by the time they reached the 4-leaf stage. Plants vernalized at the 4-leaf stage for 6 wks shifted to reproductive growth within 24 hrs of transfer to greenhouse conditions.

Table 4. Mean final leaf number and anthesis time in 6-202 vernalized at different seedling stages and vernalization periods*

Seedling Stage/ Vernalization Period (Weeks)	Final Leaf Number	Anthesis Time (DAV)
Seed		
4	9.7 a	35.9 a
5	8.3 a	34.3 a
6	8.2 a	32.6 a
Cotyledonary		
4	18.9 a	39.9 a
5	13.0 ab	32.9 a
6	10.9 b	26.9 a
4-Leaf		
4	14.0 a	32.1 a
5	12.3 a	27.5 a
6	11.6 a	26.9 a

*Values with the same letters are not significantly different at $p<0.05$. Statistical comparisons were made within each seedling stages.
DAV = days after vernalization

Figure 9. FLC expression in shoot tips of Line 6-202 as affected by duration of vernalization and plant age at which cold treatment is given. Shoot tips were collected from control non-vernalized plants at the cotyledonary, 2-leaf and 4-leaf stages. In plants vernalized at the seed and cotyledonary stages, shoot tips were collected at the end of the vernalization period (1) and when the plants have reached the 4-leaf stage. In plants vernalized at the 4-leaf stage, samples were collected at the end of the vernalization period (A) and 24 hours after transfer to greenhouse conditions (B).

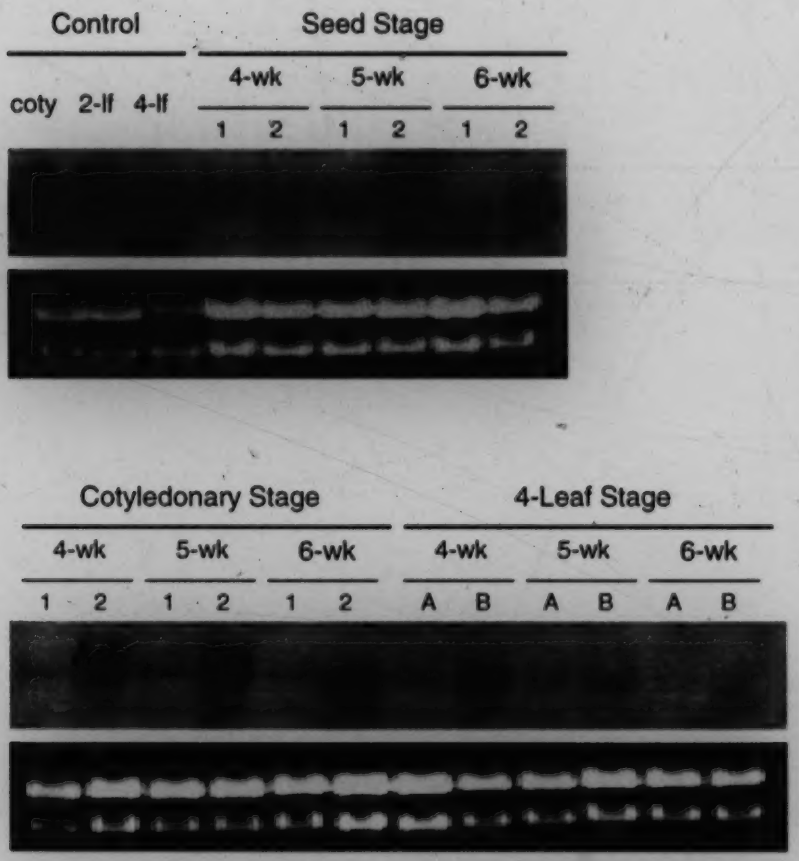
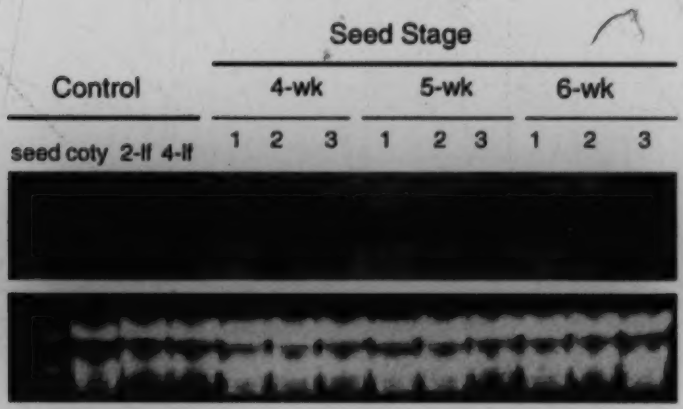


Figure 10. FLC expression in leaves of Line 6-202 as affected by duration of vernalization and plant age at which cold treatment is given. Cotyledons were collected from control non-vernalized plants at the cotyledonary stage while leaves were collected at the 2-lleaf and 4-leaf stages. In plants vernalized at the seed and cotyledonary stages, cotyledons were collected at the end of vernalization period. Leaves were then harvested when the plants have reached the 2-leaf (2) and 4-leaf (3) stages. In plants vernalized at the 4-leaf stage, samples were collected at the end of the vernalization period (A) and 24 hours after transfer to greenhouse conditions (B).



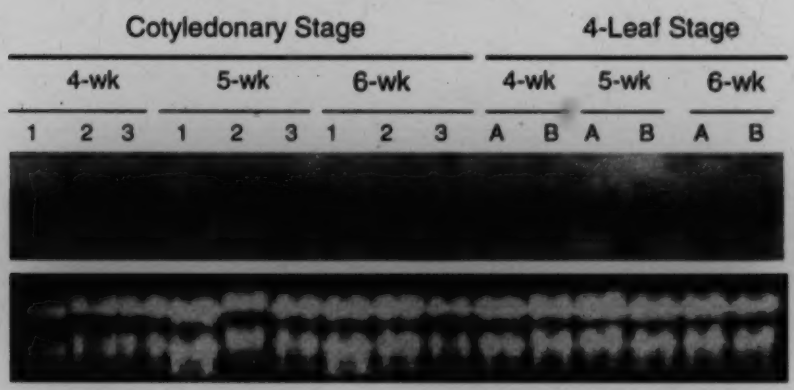
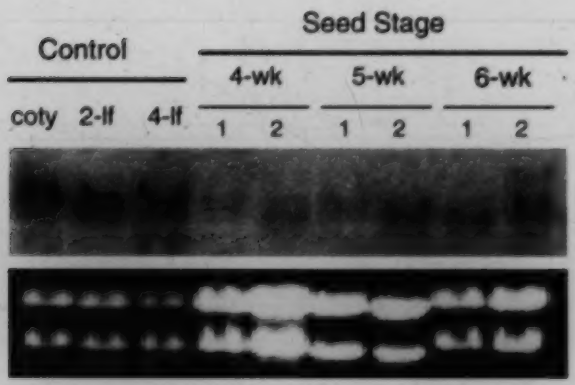
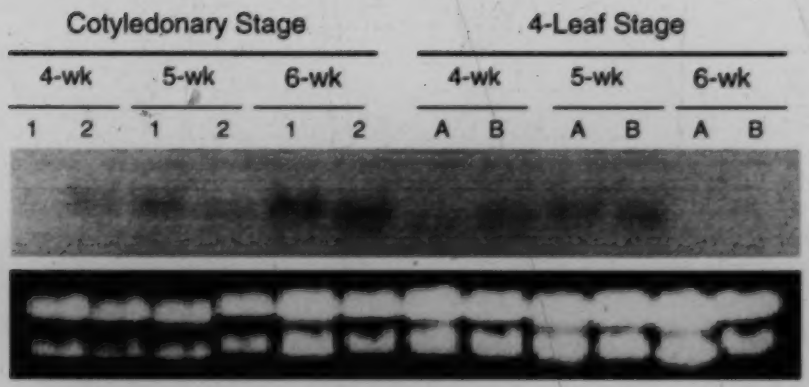


Figure 11. FLC expression in roots of Line 6-202 as affected by duration of vernalization and plant age at which cold treatment is given. Roots were collected from control non-vernalized plants at the cotyledonary, 2-leaf and 4-leaf stages. In plants vernalized at the seed and cotyledonary stages, shoot tips were collected at the end of the vernalization period (1) and when the plants have reached the 4-leaf stage. In plants vernalized at the 4-leaf stage, samples were collected at the end of the vernalization period (A) and 24 hours after transfer to greenhouse conditions (B).





4. Discussion

In *Arabidopsis*, the FLC gene was shown to have a central role in vernalization. Late-flowering ecotypes that are early-flowering when vernalized as well as late-flowering mutants that are vernalization responsive all have increased levels of FLC expression (Sheldon et al. 1999). Moreover, the down regulation of FLC levels is proportional to the duration of the cold treatment and the resulting decrease in leaf number (Sheldon et al. 2000). In the present study, FLC levels in *B. napus* were found to be higher in winter lines as compared to spring lines. This indicates that in *B. napus*, as in *Arabidopsis*, FLC also accounts for the repression of flowering that can only be removed by vernalization. As expected, FLC expression in the winter line 6-202 was down regulated by cold treatment. However, the down regulation of FLC transcript did not correlate with the quantitative flowering response of Line 6-202 seen under the different vernalization conditions. FLC expression was considerably down regulated after one week of exposure to cold treatment but the plants remained in the vegetative state. In contrast, in the late-flowering *Arabidopsis* ecotypes C24 and Pitzal, a slight decrease in FLC expression can result in as much as 30-50% reduction in leaf number (Sheldon et al. 2000). It was also observed in this study that FLC levels were the same in vernalized Line 6-202 that showed significantly different final leaf number. Taken together, these results suggest that overcoming FLC repression of flowering by cold treatment is an all or nothing process. It appears that FLC level must be down regulated to a specific level before flowering can occur. The quantitative response of Line 6-202 to vernalization is probably due to the action of other gene(s) downstream of FLC. Of the flowering time genes identified in *Arabidopsis*, the EMF gene is the most likely candidate. Two copies of the EMF gene are present in *Arabidopsis* and they both map to chromosome 5 where FLC is also located (Levy & Dean 1999, Osborn et al. 1997). EMF is a repressor of flowering that is thought to have a central role in the whole floral induction process (Haughn et al. 1995, Koornneef 1999). Lesions in this gene result in formation of floral structures with very few sessile leaves indicating that the vegetative phase has been bypassed (Sung et al. 1995, Bai & Sung 1995). Yang et al. (1995) proposed that expression of this gene is high during the early stages of vegetative growth but it decreases as the shoot matures. The transition from vegetative to reproductive phase then occurs at threshold levels of EMF. If this hypothesis holds true, it would then explain some of the results found in this study, i.e., why plants

vernalized at the 4-leaf stage flowered earlier compared to those vernalized at the cotyledonary stage.

When vernalized Line 6-202 plants were returned to the normal growing temperature of 20°C, FLC expression progressively increased as the plants grew. However, this increase in FLC expression did not inhibit flowering in the vernalized plants. This up regulation of FLC was not observed in *Arabidopsis*. In the *Arabidopsis* late-flowering ecotype C24, the decrease in FLC transcript seen at the end of the vernalization period is maintained even when the plants are transferred to 21°C (Sheldon et al. 2000). One major difference between *Arabidopsis* and *B. napus* is the genome complexity of the latter. It is believed that the modern diploid *Brassica* species descended from a hexaploid ancestor (Langercrantz 1998). As such, *B. napus* which is an allotetraploid arising from a cross between the two diploid *B. rapa* and *B.oleracea* (U 1935), has potentially six copies of the FLC gene. In *B. rapa*, three copies of the FLC gene have been identified and one FLC allele has been detected to have major effects in vernalization (Amasino, pers comm). The same situation could exist in *B. napus*. It is possible that the increase in FLC expression after cold treatment is due to the FLC copies that are not functioning in the vernalization process. It is possible that their protein products are non-functional. Alternatively, the FLC transcripts present may not actually be translated into proteins. Hawkins (1997) reported that in *B. nigra and S. arvensis*, transcripts of the cold-regulated gene BN28 were present but no protein products could be detected.

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CHAPTER V

DISCUSSION AND CONCLUSIONS

In recent years, most researches have been centred on *Arabidopsis thaliana* and this species has been developed as a model system in studying the molecular and cellular mechanisms underlying numerous plant processes. As a result of this widespread interest, a significant number of genes have been identified and isolated from this plant system (Meyerowitz & Ma 1994). Comparative mapping studies have shown that there is a high degree of collinearity between the genomes of *Arabidopsis* and *Brassica* spp. (Teutonico & Osborn 1994, Lagercrantz et al. 1996, Osborn et al. 1997). Therefore, it is possible to use the information and molecular tools developed in *Arabidopsis* to facilitate studying physiological and developmental processes in *Brassica* spp. The present study focused on studying control of floral induction in spring and winter lines of *Brassica napus* in view of what is presently known about this process in *Arabidopsis*.

In early-flowering ecotypes of *Arabidopsis*, three pathways have been identified to regulate the shift from vegetative to reproductive growth (Koornneef et al. 1998, Levy & Dean 1998). The photoperiodic induction and vernalization dependent pathways involve promotion of flowering by LD and cold treatment, respectively. The autonomous or constitutive pathway regulates flowering in the absence of external stimuli. It is dependent on the endogenous physiological state of the plant and is thought to involve GAs (Wilson et al. 1992, Michaels & Amasino 1999). The results obtained from this study together with those from previous reports (Thurling & Das 1977; Hodgson 1978; King & Kondra 1986; Murphy & Scarth 1993, 1998) strongly suggest that the same three pathways are also present in *B. napus* spring lines. As in *Arabidopsis*, the light-regulated pathway is the main pathway regulating floral induction in *B. napus* spring lines while vernalization merely hastens the flowering response. As such, the vernalization response of a spring line must always be analyzed with reference to the photoperiod conditions. This has been clearly shown in this study in two instances. First, the absence of a vernalization response under LD does not reflect the absence of the vernalization pathway but instead, it indicates that the optimal photoperiod requirement has been met. Second, the optimal vernalization period varies depending

on the photoperiod, i.e., plants grown under shorter photoperiods require longer exposures to the cold treatment to get to the minimum flowering time. Because of the precedence of the photoperiodic induction pathway, the vernalization response in *B. napus* spring lines is best measured under extreme SD conditions.

In contrast, floral induction of the *B. napus* winter lines Cascade and 6-202 was controlled solely by vernalization. These two lines failed to flower under LD without vernalization and showed no photoperiodic response when vernalized. In *Arabidopsis*, studies on the genetic control of vernalization were done using late-flowering ecotypes that flower extremely late under LD but are early flowering when vernalized (Lee et al. 1993). It has not been shown if these late-flowering ecotypes show photoperiodic response when vernalized. The vernalization requirement in the late-flowering *Arabidopsis* ecotypes was found to be controlled by the synergistic actions of two genes, FRI and FLC (Clarke & Dean 1994, Lee et al. 1994, Lee & Amasino 1995). These genes were found to function in the vernalization dependent pathway. In *B. napus* winter lines used in this study, the obligate vernalization requirement was found to have resulted from defects in the light regulated and constitutive pathways as well as the presence of the FLC gene product in the vernalization pathway. RFLP analysis indicates that the affected genes in the light regulated and constitutive pathways may be CO and FY, respectively (Osborn et al. 1997).

Results obtained from FLC expression studies in the *B. napus* winter line 6-202 indicate that FLC may be functioning differently in genotypes with an obligate vernalization requirement. In *Arabidopsis*, FLC is involved in conferring the vernalization requirement and in determining the quantitative flowering response to vernalization. FLC is highly expressed in late flowering ecotypes and mutants as compared to early flowering ecotypes (Michaels & Amasino 1999, Sheldon et al. 1999). Also, the down regulation of the FLC transcript level by vernalization is correlated with final leaf number and flowering time (Sheldon et al. 2000). In *B. napus*, FLC transcript levels were found to be higher in Line 6-202 and Cascade as compared to the spring lines. FLC, therefore, appears to confer the vernalization requirement in these winter lines. However, the quantitative flowering response of Line 6-202 to vernalization was not correlated with FLC transcript level. Instead, overcoming FLC repression of flowering by vernalization was shown to be an all or nothing process. FLC, therefore, is not

involved in the quantitative flowering response to vernalization of the *B. napus* winter line 6-202.

An interesting aspect of the response of Line 6-202 to vernalization is that plants vernalized at the cotyledonary stage required longer cold treatment than those vernalized at the seed or 4-leaf stage. Apparently, once the vegetative program has been initiated and at its early stages, activity of the genes involved in promoting vegetative growth or repressing reproductive growth are high so that longer exposures to cold treatment are required to overcome their effects. This system is, therefore, useful in studying the interaction of genes involved in the repression and promotion of flowering in *B. napus* winter lines. As has been shown in this study, although the genes identified in *Arabidopsis* late-flowering ecotypes may also be the same genes involved in vernalization in *B. napus* winter lines, the function of these genes may differ between the two plant systems. Another useful system that has been developed in this study is the early- and late-flowering Rebel lines. These two lines have been genotyped using markers linked with QTLs involved in flowering time and results indicate that they differ in just one QTL, i.e., VFN1. These lines can thus be used to facilitate identification and isolation of genes located in VFN1.

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CHAPTER VI
APPENDIX

Appendix 1. Comparative maps of *Brassica napus* (Bn), *B. rapa* (Br) and *Arabidopsis* (At) showing collinearity of QTLs and *Arabidopsis* flowering time genes. (from Osborn et al. 1997)